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DEBARYOMYCES HANSENII: A FOODBORNE YEAST THAT PRODUCES ANTI-*CANDIDA* KILLER TOXIN

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by

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Candida yeasts are commensal members of the gastrointestinal, mucosal, oral and vaginal microbiota. *Candida albicans* and *C. tropicalis* can be found as a part of the normal human commensal flora, especially in all sections of the gastrointestinal tract. However, when the host defense system and microbiota are disturbed, *Candida* can become pathogenic and cause severe infection or candidiasis. Antifungal drugs used to treat candidiasis have been shown to result in treatment failures due to drug toxicity and/or development of resistance during long term antifungal therapy and, in recent years, the incidence of *Candida* infections has increased dramatically due to the rise in the number of immunocompromised patients. Many yeast species can produce toxic proteins or glycoproteins called killer toxins or mycocins which can kill sensitive yeasts. *Debaryomyces hansenii* is the most common yeast species found in cheese, and can produce killer toxins. In order to examine the diversity and killer toxin profiles of *D. hansenii,* 48 types of cheeses were collected in 5 sampling periods for fungal isolation. Yeasts and molds were identified and 42 strains of *D. hansenii* were isolated and

screened for killer activity against *C. albicans* and *C. tropicalis* using the streak-plate agar diffusion bioassay. Killer activity of crude toxin isolated from *D. hansenii* strains was quantified at different pH values (4.5, 5, 5.5, 6.0) and temperatures (20 C, 25 C, 30 C and 35 C) by agar diffusion well bioassay. The effect of *D. hansenii* killer toxin on *C. albicans* and *C. tropicalis* growth kinetics was also studied. More than 50% of cheese examined contained the yeast species *Debaryomyces hansenii,* with *Galactomyces geotrichum* being the second most abundant yeast species, while *Penicillium roqueforti* was the most frequently isolated mold. More than 50% of *D. hansenii* strains demonstrated killer activity against *C. albicans* and *C. tropicalis*, killer toxin activity differed among the *D. hansenii* strains, and killer susceptibility differed between *C. albicans* and *C. tropicalis*. *D. hansenii* killer toxin was active against *C. albicans* up to pH 5.5 but against *C. tropicalis* to pH 6.0. Killer activity was higher at low temperature and low pH. Killer toxin activity against *C. albicans* was detected as high as 35 C. These results confirmed that the same killer toxin from *D. hansenii* can act differently in different species and correlates with temperature and pH condition; killer toxins which are active at physiological temperature may have medical application.

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Chapter 1

Literature Review

1 Cheese Introduction

Cheese is a fermented milk product with a wide range of flavors, textures and forms (Fox & McSweeney, 2004; Lucey, 2008). It is a nutritious dairy food that contains high concentrations of essential nutrients such as fat, protein, vitamins, and minerals (Konuklar et al., 2004; O'Brien & O'Connor, 2004). The protein content of cheese ranges from approximately 4-40% depending upon the cheese variety. During the cheese ripening phase, milk proteins break down into water soluble peptides and free amino acids. Hence, a significant degree of breakdown of cheese protein has occurred before it is consumed, thereby making the protein almost 100% digestible (Holland et al., 1989; O'Brien & O'Connor, 2004). The carbohydrate, lactose, in milk is fermented to lactose during cheese manufacture; any remaining lactose is lost in the whey, leaving cheese with only trace amounts of carbohydrate (O'Brien & O'Connor, 2004). Most cheeses are a significant dietary source of fat. The fat contained in cheese varies with the type of milk used and the method of manufacture. However, digestibility of the fat in different varieties of cheese is in the range of 88-94% (O'Brien & O'Connor, 2004; Renner, 1987). Most of the fat-soluble vitamins are retained in the cheese fat but concentrations of water-soluble vitamins are low due to losses in the whey (Holland et al., 1989; Renner, 1987). Additionally, cheese is an important dietary source of several minerals, especially

calcium, phosphorus and magnesium (O'Brien & O'Connor, 2004; Recker et al., 1988; Renner, 1987).

2 History

Cheese is an ancient food. It is believed that cheese making began 8000 years ago in the region of the Tigris and Euphrates Rivers. Cheese manufacture was well established in the Roman Empire and cheese was a standard ration for Roman soldiers. Migration of people throughout Europe immediately before and after the fall of the Western Roman Empire promoted further spread of cheese manufacture (Fox & McSweeney, 2004; Kosikowski & Mistry, 1997). Colonization of North and South America, Oceania and Africa by European settlers spread the consumption and manufacture of cheese throughout the world. Cheese making has been practiced for thousands of years as a cottage industry but, towards the end of nineteenth century, cheese began being manufactured in factories and technology has improved its development. Cheese is now of major economic importance in the United States, Canada and other countries (Fox & McSweeney, 2004; Kosikowski & Mistry, 1997; Sandine & Elliker, 1970).

3 Cheese Classification

There have been many attempts to develop a classification system for cheeses; however, there is no definitive classification. Different researchers have named different varieties of cheese (McSweeney et al., 2004). Sandine & Elliker (1970) classified cheese into more than 1000 varieties; a list of 1400 varieties was compiled by Jim Path (University of Wisconsin, available www.cdr.wisc.edu); and Walter and Hargrove (1972) suggested more than 400 cheese varieties. Similarly, Burkhalter (1981) classified 510 varieties of

cheese. Different authors use different methods, classifying cheese into different categories based on texture, method of coagulation or ripening indices (Fox & McSweeney, 2004; McSweeney et al., 2004; Sandine & Elliker, 1970).

3.1 Classification schemes based on texture

Schulz (1952) proposed a classification of cheese consisting of five groups based on moisture content: 1. Dried (<40% Moisture in fat-free cheese (MFFC), 2. Grated (40-49% MFFC), 3. Hard (50-59% MFFC), 4. Soft (60-69.9 % MFFC) and 5. Fresh (70-82% MFFC) (Schulz, 1952). Davis (1965) classified cheese into three categories: hard, semi-hard and soft (Davis, 1965). Almost ten years later, Walter and Hargrove (1972) classified cheese into very hard, hard, semi soft and soft, although this classification had a number of inconsistencies **(Table 1-1)** (Walter & Hargrove, 1972). Similarly, Scott (1986) classified cheese primarily on the basis of moisture content: hard, semi-hard and soft (Scott, 1986).

3.2 Classification schemes based on method of coagulation

Fox (1993) classified cheese on the basis of the coagulating agent: rennet cheese, acid cheese and heat/acid cheese. Currently, rennet-coagulated cheese represents about 75% of total cheese production; acid-coagulated cheese represents 25% of total cheese production and heat/acid cheeses are limited in number (Fox, 1993). This classification scheme has been modified by further dividing rennet-coagulated cheese varieties based on the characteristic ripening agent or manufacturing technology, such as internal bacterially ripened varieties (McSweeney et al., 2004).

Table 1-1: Classification scheme for cheeses according to Walter and Hargrove (1972)

1. Very hard (grating)
   1.1 Ripened by bacteria: Asiago, Parmesan, Romano, Sapsago, Spalen
2. Hard
   2.1 Ripened by bacteria, without eyes: Cheddar, Granular, Caciocavallo
   2.2 Ripened by bacteria, with eyes: Emmental, Gruyere
3. Semi-Soft
   3.1 Ripened principally by bacteria: Brick, Munster
   3.2 Ripened by bacteria and surface microorganisms: Limburger, Port du Salut, Trappist
   3.3 Ripened principally by blue mold in the interior: Roquefort, Gorgonzola, Danablu, Stilton, Blue Wensleydale
4. Soft
   4.1 Ripened: Bel Paese, Brie, Camembert, Hand, Neufchatel
   4.2 Unripened: Cottage, Pot, Baker's Cream, Ricotta, Mysost, Primost

4 Cheese production

The production of all varieties of cheese involves a similar approach. **Figure 1-1** summarizes the process starting with milk to the final product, cheese. However, various steps can be modified to give a product with the desired characteristics (Elisa, 2012; Fox & McSweeney, 2004). Generally, cheese manufacture begins with the selection of milk and milk coagulation. Milk consists of water, fat, carbohydrates, proteins, vitamins and minerals. Acidification of milk is achieved by using starter cultures that are added to produce lactic acid and to change the texture and flavor of the cheese during the curing

and ripening stages. The milk protein casein must be coagulated by using a coagulating enzyme or adjusting the pH to 4.6 or 5.2 in combination with heating. After the milk has coagulated the whey is removed. Salt is added to the whey-free curd, which is pressed and ripened. The ripening process varies depending upon the type of cheese desired (Elisa, 2012; Fox & McSweeney, 2004; M. Johnson & Law, 2010).

5 Microorganisms associated with cheese

Cheese harbors a complex microbial ecosystem, including bacteria, yeast and molds (Arfi et al., 2005). These microorganisms may either contribute (directly or indirectly) to, or detract from, the organoleptic qualities of the finished product by releasing enzymes into the cheese matrix through autolysis (Beresford & Williams, 2004). Generally, microorganisms gain entry into the cheese either by addition as part of the starter culture or through association with the ingredients used in the cheese production such as air, farm workers, cheese makers, brine and cheese making equipment (Beresford & Williams, 2004; Borelli et al., 2006; Mounier et al., 2006; Pereira-Dias et al., 2000; Vacheyrou et al., 2011).

A number of factors control the growth of microorganisms in cheese, including water content, salt concentration, pH, organic acids, redox potential and ripening temperature. These factors play an important role in survival, growth and proliferation of microorganisms in the cheese (Beresford & Williams, 2004; Beresford et al., 2001).

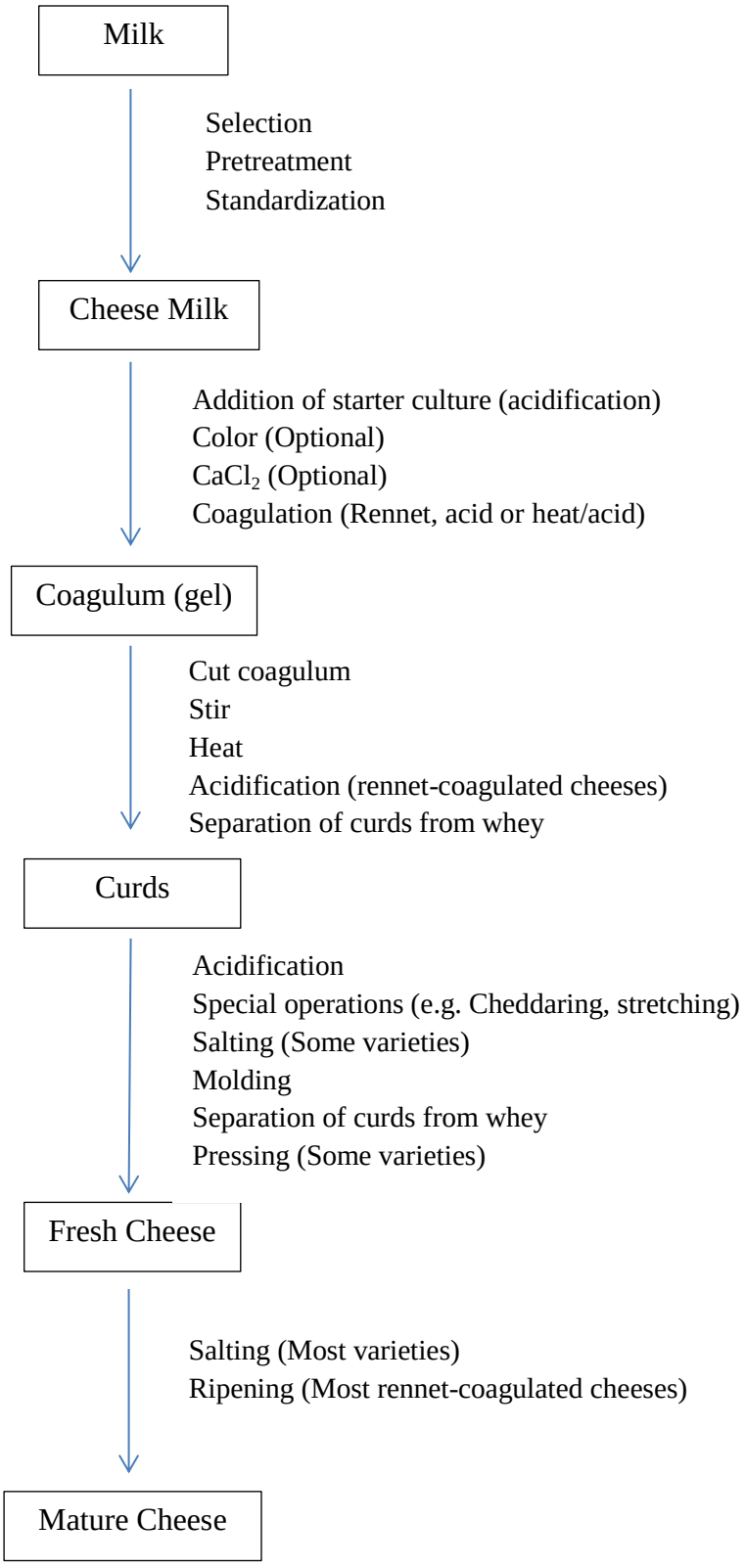

Figure 1-1. General cheese manufacture (Fox et al., 2000)

5.1 Moisture

All microorganisms require water; the availability of water determines the growth of the microorganisms. An increase in the moisture content of cheese leads to an increase in the microbial growth. Water activity (a_w) is directly proportional to the moisture content of the cheese and inversely proportional to the concentration of NaCl. It also contributes to the control of microbial metabolic activity and multiplication (Beresford et al., 2001). During the first stage of cheese manufacture, the a_w is high, supporting the growth and activity of the starter culture. However, in the ripening stage, a_w decreases due to water loss by evaporation, salt and hydrolysis of proteins and triglycerides (Cogan, 2000). Values of a_w vary in different zones of a cheese (Beresford et al., 2001).

5.2 Salt

Salt and a_w are interrelated, affecting the microbial growth in cheese. The a_w decreases with an increase in salt concentration, resulting in a major inhibitory factor for microbial growth. The salt concentration in cheese ranges from 0.7-7 g/100 g, corresponding to a_w values of 0.99-0.95, respectively (Cogan, 2000). Therefore, the microbial population and diversity differs with the salt concentration among cheeses.

5.3 pH and organic acids

pH and organic acids play an important role in the growth and survival of many microorganisms. As organic acids, such as lactic acid and propionic acid, accumulate in the course of bacterial fermentation, the pH is lowered, suppressing the growth of acid-sensitive species (Beresford et al., 2001).

5.4 Ripening temperature

The microorganisms involved in starter cultures in cheese manufacture and ripening are either mesophilic or thermophilic. These bacteria grow optimally at temperatures between 20-39 C and 40-53 C, respectively. Temperature can limit the growth of the secondary flora and prevent the growth of potential spoilage and pathogenic organisms (Beresford et al., 2001).

5.5 Redox potential

The redox potential of cheese is one of the major factors determining the type of microorganisms that can grow (Beresford et al., 2001). Fermentation of lactose by the starter culture during manufacture reduces a small amount of O_2 in the milk to water; which represses the growth of aerobic organisms (Crow et al., 1995).

6 Cheese Microorganisms

Microorganisms are an essential component of all cheese varieties. They can be divided into two groups.

6.1 Primary culture

Cheese cannot be made without the use of certain species of lactic acid bacteria (LAB). LAB produce lactic acid from the lactose in the milk during manufacture. These bacteria, introduced early in cheese manufacture, are called primary cultures or starter bacteria (Chamba & Irlinger, 2004; Parente & Cogan, 2004). Starter culture bacteria are capable of producing sufficient acid to reduce the pH of milk rapidly and coagulate the milk. *Lactococcus, Streptococcus thermophilus, Lactobacillus delbrueckii* and *L. helveticus* are commonly used starter bacteria (Beresford & Williams, 2004). In addition to the starter

culture, uninoculated adventitious microorganisms can participate in cheese ripening (Swearingen et al., 2001).

6.2 Secondary Microflora

In addition to the starter culture, a secondary microflora can participate in the ripening of cheese (Swearingen et al., 2001). These microorganisms include organisms which do not have a role in acid production but play an important role in organoleptic and biochemical changes in or on the surface of the cheese (Chamba & Irlinger, 2004; Parente & Cogan, 2004). This microflora originates from the milk, cheese making utensils or the factory environment. The secondary microflora includes many yeasts (*Geotrichum candidum, Debaryomyces hansenii*), molds (*Penicillum camemberti, P. roqueforti*) and bacteria other than the primary culture LAB (Chamba & Irlinger, 2004). Over 40 non-starter yeast and mold species have been isolated from cheese.

6.2.1 Molds

Molds are filamentous fungi that reproduce by the production and dispersal of spores. A spore germinates, producing hyphae, which branch to form a mass of mycelium. This mycelium can be visible on the surface of the food (Hocking, 1997).

Molds are used as adjuncts in mold surface-ripened soft cheese (Brie, Camembert, or goats's milk cheese) and blue-veined cheeses (Bavarian Blue, Bleu d'Auvergne, Bleu des Causses and Roquefort, Cabrales, Gorgonzola, Danablu and Stilton) (Chamba & Irlinger, 2004). Molds used as adjuncts in cheese play important roles in the appearance of the cheese surface and body. Molds such as *Penicillim camemberti* and *P. roqueforti* are able to utilize lactic acid. Their growth leads to an increase in pH and proteolysis of the cheese

which makes cheese soft (Chamba & Irlinger, 2004; Gripon, 1993). Molds have lipolytic activity and can produce aroma, and interact with other microorganisms (Chamba & Irlinger, 2004).

Most cheeses have a high concentration of protein, fat and volatile fatty acids and low pH due to lactic acid production by LAB. Similarly, water activity is low due to a high salt concentration. These conditions make cheese a good substrate for the growth of certain molds (Beresford & Williams, 2004). Mold growth can occur on cheese during its ripening period or in the production chain (Taniwaki & Van Dender, 1992). However, most cheeses are refrigerated, reducing the range of colonizing mold species (Hocking, 1994). Molds associated with different cheeses may originate from ingredients used in the cheese production, air, farm workers, cheese makers, brine, and cheese making equipment (Beresford & Williams, 2004; Borelli et al., 2006; Mounier et al., 2006; Pereira-Dias et al., 2000).

6.2.1.1 Mold species isolated from cheese

Due to the distinctive characteristics of each kind of cheese, the mold species isolated from different cheeses differ. Commonly, mold isolated from cheese includes *Penicillium, Aspergillus, Cladosporium*, *Mucor* and *Trichorderma*. (Chamba & Irlinger, 2004) **(Table 1-2)**.

Table 1-2: Molds isolated from various cheeses

Cheese	Place	Mold isolated	Reference
May Bryndza	Slovakia	*Alternaria alternata, Aspergillus fumigatus, Cladosporium cladosporioides, Penicillium aurantiogriseum, P. camemberti, P. freii, P. polonicum, P. viridicatum, Fomes fomentarius, Gymnoascus reesii, Chaetomium globosum, Sordaria alcina,*	(Pangallo et al., 2014)
Cheddar	Argentina	*Phoma glomerata, Penicillium commune, P. chrysogenum, P. glabrum, P. brevicompactum, P. crustosum, Mucor hiemalis, Monilliclla suaveolens*	(Basílico et al., 2001)
Taleggio	Italy	*Penicillium commune, Cladosporium oxysporum, C. cladosporioides, Aureobasidium pullulans, Eutypella scoparia,*	(Panelli et al., 2012)
Jarlsberg	Norway	*Aspergillus fumigatus, Eurotium herbariorum, Penicillium aurantiogriseum, P. commune, P. crustosum, P. echinulatum, P. expansum, P. palitans, P. roqueforti, P. solitum, P. viridicatum, Phoma* sp.	(Kure et al., 2001)
Norvegia	Norway	*Acremonium strictum, Cladosporium cladosporioides, Mucor hiemalis, M. plumbeus, M. racemosus, Penicillium aurantiogriseum, P. brevicompactum, P. commune, P. crustosum, P. echinulatum, P. expansum, P. palitans, P. roqueforti, P. solitum, P. spinulosum, P. viridicatum, Phoma* sp.,	(Hocking & Faedo, 1992; Kure et al., 2001)

Cheddar		*Cladosporium cladosporioides, C. herbarum, Penicillium commune, P. glabrum, Phoma* spp.	(Hocking & Faedo, 1992)
Hard, semi hard and semi soft	Europe	*P. commune, P. nalgiovense, P. verrucosum, P. atramentosum, P. solitum, P. chrysogenum, P. roqueforti, P. crustosum ,P. echinulantum, Aspergillus versicolor,*	(Lund et al., 1995)
Soft ripened, washed rind, natural rind, Ripened in the mass and mixed	Canada	*Aspergillus fumigatus, Aureobasidium pulullans, Cladosporium sp. C. cladosporioides, Cochliobolus sativus, Eurotium niveoglaucum, Eurotium* sp,*. Fusarium domesticum, Lecythophora hoffmannii, Lichtheimia corymifera, Mucor circinelloides, M. racemosus, Peyronellaea glomerata*	(Lavoie et al., 2012)

6.2.2 Yeasts

Yeasts are unicellular fungi that reproduce by budding or fission. Because of their wide physiological diversity, yeasts are able to grow in a broad range of habitats (Jacques & Casaregola, 2008). Dairy products provide a unique ecological niche for the growth of specific yeasts (Fleet, 1990).

6.2.2.1 Yeasts in Cheese

The occurrence of yeasts in cheese is not unexpected as cheese has various properties that encourage the proliferation of yeasts such as high acidity, storage at low temperature, low moisture content and high salt concentration (Viljoen, 2001). Yeasts play diverse roles in the quality and safety of cheese (El-Sharoud et al., 2009; Jacques & Casaregola, 2008) and their presence is of major importance as they can have beneficial or detrimental effects (Beresford et al., 2001).

Yeasts contribute directly or indirectly to the appearance of cheese (Chamba & Irlinger, 2004). Some yeasts show varied ability to metabolize substrates such as lactate and citrate. Breakdown of these substrates results in de-acidification on the cheese surface and an increase in pH, stimulating the growth of molds and bacteria (Eliskases-Lechner & Ginzinger, 1995). Yeasts show proteolytic and lipolytic activity; however, this activity differs between species and strains. *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Kluyveromyces marxianus* and *Debaryomyces hansenii* exhibit a large diversity in proteolytic and lipolytic activity (Chamba & Irlinger, 2004; Lucia et al., 2001). Some yeasts have been shown to contribute to the development of different flavors

during cheese ripening. Flavors are a result of the production of ethanol, aldehydes, esters, and the degradation of amino acids to ammonia and keto acids (Fleet, 2007; Spinnler et al., 2001). Yeasts also have the ability to interact with other microorganisms and can reduce the occurrence of undesirable molds such as *Aspergillus, Mucor* and *Penicillium* species (Chamba & Irlinger, 2004). In addition to these beneficial factors of yeast, some species have detrimental effects. Typical defects due to unwanted yeast presence include spoilage, gas production, off-flavors, discolorations and changes in texture (Jakobsen & Narvhus, 1996; Rohm et al., 1990).

Yeasts colonize numerous types of cheese during the early stages of cheese making. Yeast colonization is higher on the outer surface than the inner surface (Chamba & Irlinger, 2004). In traditional cheeses, the source of the yeasts in cheese came from raw milk, utensils, and the cheese factory environment (Zambonelli et al., 1996). Modern cheese factories may use yeasts as adjuncts; they are added to the milk (Chamba & Irlinger, 2004). However, environmental species and adjuncts vary with the type of cheese and factory, making the presence of yeast variable among types of cheese (Viljoen et al., 2001).

6.2.2.2 Yeast species isolated from cheese

Due to the different characteristics of each kind of cheese, a variety of yeast species has been isolated from different types of cheese (Jakobsen & Narvhus, 1996). The species of yeasts isolated most frequently from cheeses are listed in **Table 1-3**, and **Table 1-4** shows yeasts isolated from particular types of cheese.

Table 1-3: Yeast species isolated from cheese (from Jacques & Casaregola, 2008)

Common yeast species	Rarely occurring yeast species
Geotrichum candidum	*Saccharomyces unisporus*
Debaryomyces hansenii	*Saccharomyces exiguus*
Kluyveromyces marxianus var. *lactis*	*Dipodascus capitatus*
Kluyveromyces marxianus var. *marxianus*	*Pichia fermentans* var. *fermentans*
Candida zeylanoides	*Pichia kluyverii* var. *kluyverii*
Candida catenulata	*Pichia membranifaciens*
Saccharomyces cerevisiae	*Pichia pseudocactophila*
Candida intermedia	*Candida rugosa*
Yarrowia lipolytica	*Candida sake*
Torulaspora delbrueckii	*Candida tenuis*
	Pichia jadinii
	Dipodocus capitatus
	Candida versatilis
	Issatchenkia occidentalis
	Clavispora lusitaniae
	Zygosaccharomyces rouxii
	Williopsis californica

Table 1-4: Yeasts isolated from specific cheese types

Cheese	Place	Yeast isolated	Reference
May Bryndza	Slovakia	*Candida xylopsoci, C. inconspicua, Debaromyces hansenii, Galactomyces candidus, Kluyveromyces marxianus, Pichia kudriavzevii, Yarrowia lipolytica, Trichosporon lactis*	(Pangallo et al., 2014)
Oscypek	Poland	*Candida pararugosa, C. zeylanoides, Geotrichum silvicola, Saccharomyces* spp., *Kluyveromyces marxianus, Debaryomyces hansenii*	(Alegria et al., 2012)
Soft ripened, washed rind, natural rind, ripened in the mass and mixed	Canada	*Candida catenulata, C. parapsilosis, C. pararugosa, C. tropicalis, C. zeylanoides, Cryptococcus diffluens, Cr. curvatus, Issatchenkia orientalis, Kluyveromyces marxianus, Pichia jadinii, P. fermentans, P. membranifaciens, Rhodotorula glutinis, R. mucilaginosa, Saccharomyces servazii, Trichosporon aquatile, T. asahii, T. domesticum, T. jirovecii*	(Lavoie et al., 2012)
Fresh Domiati cheese, stored Domiati cheese and Kariesh cheese	Egypt	*Issatchenkia orientalis, Candida albicans, C. catenulata, Clavispora lusitaniae, Kodamaea ohmeri, Kluyveromyces marxianus,*	(El-Sharoud et al., 2009)
Water buffalo Mozzarella	Italy	*Pichia pastoris, P. barkeri, P. norvegensis, Clavispora lusitaniae, Candida pararugosa, C. sorbophila, C. butyri, Kluyveromyces marxianus, Saccharomyces cerevisiae*	(Aponte et al., 2010)

Soft smear-ripened Taleggio	Italy	*Debaryomyces hansenii, Torulaspora delbrueckii, Kluyveromyces lactis, K. marxianus, Pichia guilliermondii, Yarrowia lipolytica, Candida sake, C. etchellsii*	(Giannino et al., 2011)
Sardinian ewe's cheeses		*Candida catenulata, C. sake, Debaryomyces hansenii, Dekkera anomala, Geotrichum candidum, Kluyveromyces lactis, K. marxianus, Pichia fermentans, P. membranaefaciens, Rhodotorula rubra, Yarrowia lipolytica*	(Cosentino et al., 2001)
Smear-ripened cheeses		*Clavispora lusitania, Debaryomyces hansenii, Geotrichum candidum, Kluyveromyces lactis K. marxianus, Yarrowia lipolytica*	(Gente et al., 2007)
Fiore Sardo		*Debaryomyces hansenii, Kluyveromyces lactis, K. marxianus, Geotrichum candidum, Candida zeylanoides, C. lambica, C. lipolytica, C. rugosa, C. magnolia, C. lusitaniae, Monilella suaveolens, Cryptococcus curvatus, Trichosporon cutaneum, Rhodotorla rubra, Torulaspora delbrueckii, Saccharomyces cerevisiae, Zygosaccharomyces bisporus, Pichia etchelsii*	(Fadda et al., 2004)
Gouda Cheese	South Africa	*Candida catenulata, C. laurentii, C. zeylanoides, Cryptococcus albidus, Debaryomyces hansenii, Kluyveromyces marxianus, Rhodotorula glutinis, R. minuta, Saccharomyces cerevisiae, Sporobolomyces roseus, Torulaspora delbrueckii, Trichosporon beigelii, Yarrowia lipolytica*	(Welthagen & Viljoen, 1998)
Feta	Greece	*Kluyveromyces lactis, Pichia membranifaciens, P. fermentans, Candida zeylanoides*	(Rantsiou et al., 2008)

7 *Debaryomyces hansenii*

Debaryomyces hansenii, the sexual stage (teleomorph) of *Candida famata* (Krcmery & Kunova, 2000), is widespread in nature and common in cheeses (Borelli et al., 2006; Cosentino et al., 2001; Vasdinyei & Deák, 2003) and food products with high salt concentrations (Del Bove et al., 2009; Prista et al., 2005). It is abundant in different types of cheese due to its ability to grow in the presence of high salt (Prista et al., 2005), and low pH, (Capece & Romano, 2009) and its ability to metabolize lactic and citric acids (Ferreira & Viljoen, 2003). It produces proteolytic and lipolytic enzymes that can metabolize milk proteins and fats as well as contribute to the cheese ripening process (Roostita & Fleet, 1996).

Similarly, *D. hansenii* is frequently detected in gut microflora from individuals who consume cheese. Older reports implicate *D. hansenii* in human fungemia; however, *D. hansenii* is extremely difficult to differentiate from *Pichia* (*Candida*) *guilliermondii* on the basis of phenotype (Nishikawa et al., 1996), leading to frequent errors in identification. *Pichia gulliermondii* (*Candida guilliermondii*) is widely distributed in nature and a common constituent of the normal human microflora. Globally, this species accounts for 1-2% of all candidemia (Lan & Xu, 2006). *D. hansenii* is no longer believed be an important human pathogen as previously thought (Desnos-Ollivier et al., 2008).

D. hansenii is a haploid yeast that reproduces vegetatively by multilateral budding while sexual reproduction proceeds via heterogamous conjugation (Johnson & Echavarri-Erasun, 2011). It can grow at high osmotic concentrations - up to 25% NaCl and 18%

glycerol (Butinar et al., 2005). Besides having a halotolerant character, these yeasts show a broad spectrum of carbon substrate assimilation (Yadav & Loper, 1999). In addition, *D. hansenii* also has the ability to synthesize killer toxins (Buzzini & Martini, 2001).

8 Killer yeast phenomenon

Yeasts can produce toxic proteins or glycoproteins called killer toxins, which can lead to death of sensitive yeast isolates (Schmitt & Breinig, 2002). Killer activity has been reported in more than 100 yeast species belonging to more than 20 genera, and killer character does not appear uniformly among either within a species or in relation to the sources of isolation (Buzzini & Martini, 2001; Young & Yagiu, 1978). Killer genetic systems can be cytoplasmic (either dsRNA virus-like particles or linear DNA plasmids) or chromosomal.

8.1 Killer toxin mode of action

Depending on the producing species and the toxin, killer activity against sensitive yeasts occurs in one of several different general modes. In one of the best-studied cases, *Saccharomyces cerevisiae* K1 toxin, activity first occurs with binding the sensitive cell at a cell wall receptor (β-1,6-D-glucan) and plasma membrane receptor (Kre1p) (Breinig et al., 2002) and damages the membrane by forming of ion channels with the concomitant release of K^+ ions, ATP and other metabolites. Formation of a trans-membrane channel destroys the pH gradient of cell by modification of the ion gradient at the cell-environment interface of the membrane and results in sensitive cell death (de la Pena et al., 1981). However, this mechanism of formation of ion channels is poorly understood (Starmer & Lachance, 2011).

Some killer toxins initially bind to α-1, 3-linked mannose residues of the cell wall and receptor of plasma membrane, enter into the cells by endocytosis, then enter the nucleus and halt cell division at the early G2 stage, inhibiting DNA and β-1, 3-glucan synthesis (Eisfeld et al., 2000; Starmer & Lachance, 2011).

The yeast species *Saccharomyces cerevisiae* produces several killer toxins, including K1, K2 and K28, which are encoded by different cytoplasmically-inherited, dsRNSA virus-like particles; additional killer toxins KHR and KHS are chromosomally encoded (Goto et al., 1991). These toxins differ in mode of action (Breinig et al., 2002). K1, KHR and KHS toxins form ion channels (Breinig et al., 2002; de la Pena et al., 1981; Goto et al., 1991) but toxin K28 inhibits cell division in the G2 phase (Eisfeld et al., 2000). Toxin produced by *Kluyveromyces lactis* is cytoplasmically inherited and inhibits cell division at G2 stage (Butler et al., 1991; Gunge, 1995). Characterization of the killer toxins produced by *Pichia s*pecies suggests that they are both produced by linear DNA plasmids (Klassen et al., 2001; Worsham & Bolen, 1990) and encoded by nuclear genes (Kagan, 1983). Regardless, toxin activity seems to increase membrane permeability and cause the loss of ions (Suzuki et al., 2001), and inhibit β-1, 3-glucan synthesis (Santos et al., 2000). *Williopsis* species make four toxins (HM-1, K-500, WmKT and Wicaltin) (Yamamoto et al., 1986) targeting β-glucans in the cell wall, resulting in cell death (Theisen et al., 2000). The *Williopsis* killer genetic system is not clearly understood. The killer protein produced by *Schwanniomyces occidentalis* is chromosomally encoded (Chen et al., 2000; Starmer & Lachance, 2011).

Killer toxins differ between species or strains, showing diverse characteristics in terms of genetic location, molecular size, mature structure of the protein and mode of action

(Kagan, 1983; Starmer & Lachance, 2011). Additionally, yeast killer toxin activity is dependent on pH and temperature, (Rosini, 1983).

8.2 *Debaryomyces hansenii* killer toxin

D. hansenii produces active killer toxin as cells progress from exponential to early stationary phase and optimal stability is observed at pH 4.5 and temperature 20°C. Above 4.5 and 20 C killer toxin activity decreases (Marquina et al., 2001). *D. hansenii* has been shown to carry various nuclear genes derived from plasmids pDHL1, pDHL2 and pDHL3 which are not essential for killer toxin production (Gunge et al., 1993; Satwika et al., 2012); however, the linear plasmid pDHL1 from *D. hansenii* is homologous to killer plasmid pGKL1 from *Kluyveromyces lactis* (Fukuda et al., 1997). *D. hansenii* killer toxin is a secreted protein with low molecular weight encoded by chromosomal genes. The mechanism underlying the action of killer toxins produced by *D. hansenii* is not yet well understood. The killer toxin is initially adsorbed by (1-6)-β-D-glucan (Santos et al., 2002), but the detail of the killing mechanism is unknown. We still do not know how the killing activity occurs, whether by ion leakage or inhibition of DNA synthesis or cell cycle arrest in G1, or inhibition of glucan synthesis, or by other means lacking analogy in previously described killer systems. Additionally, *D. hansenii* killer toxin has been studied in few strains, and it is unknown whether additional toxins and modes of action may exist, as is the case in *S. cerevisiae*, *Williopsis* and other yeasts.

9 Pathogenic Yeasts

Yeasts are the most prominent disease-causing fungi. About 50 species of yeast are associated with fungal diseases, the most common of which are *Candida* spp,, *Cryptococcus neoformans* and *Cr. gattii* (Chester & Cooper, 2011).

9.1 *Candida* species

Candida are eukaryotic diploid yeasts, ability to grow polymorphically either in the form of budding yeasts or filaments (hyphae) (Moran et al., 2012; Williams et al., 2013). This group comprises of approximately 200 species (Akpan & Morgan, 2002). *Candida* species are very versatile organisms and have the ability to grow in a diverse range of environmental niches (Moran et al., 2012) including air, water and foodstuffs (Cooper, 2011). However, a relatively small number of *Candida* species are particularly associated with colonization in the human mucosa, oral cavity, gastrointestinal tract and/or vagina and cause disease **(Table 1-5)** (Moran et al., 2012; Perlroth et al., 2007).

Candida species are detectable in humans by the first month of life (Kumamoto & Vinces, 2005) and start to colonize the mucosal surfaces such as the oral, respiratory and vaginal cavities and the intestinal tract (Perlroth et al., 2007). The GI (gastrointestinal) tract is home to a variety of *Candida* species due to favorable environmental conditions such as pH, oxygen levels and nutrients (Odds, 1987; Soll et al., 1991). *Candida* species are detectable in all GI tract sections of healthy humans but the population differs in each section. Normally, *Candida* species range from $0\text{-}10^2$ CFU/ml of intestinal contents in the stomach and jejunum but reach higher counts in the ileum and colon, ranging from $10^2\text{-}10^6$ CFU/ml of intestinal contents (Schulze & Sonnenborn, 2009).

Table 1-5: Medically relevant *Candida species (*from Calderone & Clancy 2012*)*

Frequency	**Organism**
Common	*C. albicans*
	C. glabrata
	C. parapsilosis
	C. tropicalis
Infrequent	*C. krusei*
	C. dubliniensis
	C. guilliermondii
	C. lusitaniae
	C. rugosa
	C. orthopsilosis
	C. metapsilosis
Rare	*C. famata*
	C. inconspicua
	C. kefyr
	C. lipolytica
	C. norvegensis
	C. sake
	C. zeylanoides

Host immune system and competitive functions of the normal microbiota of skin, mucosal and intestinal surface are critical factors for *Candida* colonization (Standaert-Vitse et al., 2009). Most of the time, *Candida* colonization on mucosal surfaces or the GI tract is contained and tolerated (Goldman & Huffnagle, 2009) by both specific (immune system) and non-specific (intestinal flora, peristalsis, and intestinal enzymes) host defense systems (Piispanen & Hogan, 2008). Therefore, *Candida* species behave like harmless commensals in normal healthy humans (Kumamoto & Vinces, 2005; Perlroth et al., 2007). However, under certain conditions such as the disruption of normal immune response of the host or alteration in composition of GI microbiota, *Candida* becomes pathogenic and can cause oral, cutaneous, or systemic infection, commonly known as candidiasis (Goldman & Huffnagle, 2009; Hube, 2004). Therefore *Candida* is considered an opportunistic pathogen (Moran et al., 2012; Walker et al., 2009).

Candida species can cause two major types of infections in humans: infections on the mucosal surface, and life-threatening systemic infection (Mayer et al., 2013; Tsai et al., 2013). Oropharyngeal candidiasis (OPC), vulvovaginal candidiasis (VVC) and infections in the gastrointestinal epithelial cells are common infections on mucosal surfaces. In addition to causing mucosal infections, *Candida* species can also cause systemic and invasive infections and have emerged as the fourth most common cause of blood-borne infection in the United States (Mayer et al., 2013; Moran et al., 2012; Tsai et al., 2013). More than 90% of these infections are caused by *Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* (Perlroth et al., 2007). Increasing populations of immunocompromised patients, patients using intravenous catheters, patients on total

parenteral nutrition; increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies, and transplantation contribute the increase of *Candida* infections (Ortega et al., 2011).

9.1.1 *Candida albicans*

C. albicans is the most commonly recovered *Candida* species worldwide, representing a global average of 66% of all *Candida* species isolated from humans. However, the frequency of *C. albicans* varies around the world (Lim et al., 2012; Pfaller & Diekema, 2007). It is recovered from a broad range of human anatomical locations including the oral mucosa, the gut, the vaginal mucosa and the skin (Moran et al., 2012) and is well adapted to mucosal surfaces (Moran et al., 2012). But *C. albicans* is an opportunistic pathogen which causes infections on mucosal surfaces and systemic infections. It is the most common cause of OPC (Vargas & Joly, 2002), VVC (Sobel, 1998), systemic and invasive candidiasis (IC) (Moran et al., 2012). *C. albicans* is generally estimated to cause approximately 50-60% of all IC cases (Perlroth et al., 2007).

9.1.1.1 Virulence factors

C. albicans pathogenicity is supported by a wide range of virulence factors such as adhesins, secreted hydrolytic enzymes, and morphology switching (Lim et al., 2012; Mayer et al., 2013; Moran et al., 2012; Tsai et al., 2013). These virulence factors aid in invading the host and result in infections.

9.1.1.1.1 Adhesins

Adherence to the host is the initial and critical step in microbial infections and is a complex and multifactorial process (Williams et al., 2013). The outermost layer of *C.*

albicans contains diverse carbohydrates and proteins that contact host proteins in epithelial and endothelial cells. Adherence of *Candida* is mediated by specific molecules called adhesins on the fungal cell surface which interact with specific ligands on the host cell surface. The agglutinin-like sequence (ALS) protein family contains the most recognized adhesins (Bowman & Free, 2006; Hoyer & Hecht, 2001; Tsai et al., 2013) and is comprised of eight ALS genes (Als1-Als7, and Als9) which are associated with the β-1, 6-glucan of *C. albicans.* The N terminus of ALS protein, which contains a signal sequence, an immunoglobulin-like domain and a threonine-rich domain, is involved in ligand binding on the host surface (Hoyer & Hecht, 2001; Williams et al., 2013). Other proteins involved in *C. albicans* adhesion include hyphal wall protein 1 (HWP1), enhanced adherence to polystyrene (EAP1) and integrin analog (INT1) (Williams et al., 2013).

9.1.1.1.2 Hydrolytic enzymes

C. albicans can generate a number of hydrolytic enzymes with broad substrate activity that can help in nutrient acquisition, damage host cells and facilitate dissemination within the host (Tsai et al., 2013; Williams et al., 2013). Three different classes of secreted hydrolases are expressed by *C. albicans*: proteases, phospholipases and lipases.

Ten different secreted aspartate proteinases (SAP1-SAP10) are known for *C. albicans*. Depending on the situation, different tissue-specific SAPs are induced and these are important for the invasion of tissues and organs during various stages of *C. albicans*-host interactions (Hube, 2004; Mayer et al., 2013; Schulze & Sonnenborn, 2009; Tsai et al., 2013; Williams et al., 2013). SAP1-3 is highly expressed in yeast cells and SAP4-6 is expressed in hyphal cells (Naglik et al., 2004). SAP7 and SAP8 appear in some clinical

samples of human oral infections and vaginal infection, respectively (Tsai et al., 2013). SAP9 and SAP10 are expressed in both yeast cells and hyphae (Hube & Naglik, 2001).

The four secreted phospholipases A to D (PLA, PLB, PLC and PLD) hydrolyze one or more ester linkages of glycerophospholipids on the host cell membrane, and are critical factors in tissue invasion. Among these phospholipases, PLB represents the major activity, with broad substrate specificity and hydrolase activity (Yang, 2003). In addition to SAPs and phospholipases, *C. albicans* also secretes lipases which hydrolyze the ester bonds of mono-, di-, and triacylglycerols (Schaller et al., 2005).

9.1.1.1.3 Morphology switching

C. albicans is a polymorphic fungus which can switch its form from the unicellular yeast to the filamentous hyphal or presudohyphal form. The ability of *C. albicans* to switch between the yeast and filamentous forms is important for virulence (Lim et al., 2012). Phenotypic switching helps *C. albicans* adapt to the changing environment at different anatomic loci in the host (Tsai et al., 2013). Morphology switching makes it difficult for the immune system to recognize *C. albicans* and helps with biofilm formation (Schulze & Sonnenborn, 2009).

9.1.1.1.4 Biofilms

The *C. albicans* biofilm consists of a complex, organized structure of yeast and filamentous cells enclosed in a self-produced extracellular matrix (Chandra et al., 2001). The biofilm has higher resistance than planktonic cells to antifungal drugs and host immune response (Ramage et al., 2005).

9.1.2 *Candida tropicalis*

C. tropicalis is a diploid, clinically important pathogenic yeast. It is found as part of the normal commensal flora in humans and can cause invasive candidiasis (Leung et al., 2002). *C. tropicalis* causes disseminated infection in neutropenic patients and infections are commonly acquired from normal commensal flora from host intestinal tract (Leung et al., 2002; Richardson & Lass-Florl, 2008). Cancer patients suffer more frequently from *C. tropicalis*; and *C. tropicalis* infections result in higher mortality than infection caused by other species (Muñoz et al., 2011). It is rarely associated with oropharyngeal infection but is more virulent in patients with hematologic malignancies, and causes disseminated infection with higher mortality rate (Leung et al., 2002).

Infection caused by *C. tropicalis* varies widely with geographical area. *C. tropicalis* infection is higher in South America, Middle East and Southeast Asia than North America and Europe. The reason for these differences is not clear (Muñoz et al., 2011).

9.2 Anti-Candida activity of yeast killer toxin

Some killer toxins have broad antifungal and antimicrobial effects. The killer toxins from *Williopsis subsuffciens, W. beijerinckii, W. mrakii, Hanseniaspora uvarum,* and *Hansenula anomala* have anti-*Candida* activity (Mathews et al., 1998; Schmitt et al., 1997). Killer phenomenon of yeasts plays an important role in the prevention and control of pathogenic yeasts (Starmer & Lachance, 2011). The toxin produced by *Lindnera mrakii* has activity against *Candida* species (Walker et al., 1995). *Williopsis mrakii* (NCYC 500) showed extensive anti-*Candida* activity producing killer toxin K-500 (Hodgson et al., 1995) but the exact activity of other killer toxins HM, WmKT and

Wicaltin against *Candida* species is unclear. *Zygosaccharomyces bailii* killer toxins kill *C. albicans* by disrupting the membrane and unbalancing the ion gradient (Weiler & Schmitt, 2003). More recently, killer toxin produced by *Wickerhamomyces anomalus* has shown killer activity against *C. albicans* at pH 3.5 and 16 C (Guo et al., 2013). Crude killer toxin produced by *D. hansenii* P41 was tested at 37 C against different Candida species and showed 100% killer activity against C. *glabrata*, *C. haemulonii, C. inconspicua,* and *C. parapsilosis* (Buzzini et al., 2004). The *D. hansenii* killer toxin showed killer activity against sensitive strain *C. boidinii* IGC 3430 isolated from olive brines (Marquina et al., 1992).

From this information we can conclude that some yeast species have anti-*Candida* killer activity and their activity depends upon the growing medium, temperature and pH. However, there are still many open and unresolved questions regarding killer toxins of *D. hansenii* such as, which genetic system produce killer toxins, do they produce only one killer toxin or several, the detailed mechanism of mode of action, and killer activity against different gut *Candida* species **Table 1-6**.

Table 1-6: Characteristics of the yeast killer toxins (from Marquina et al., 2002)

Yeast species	Killer toxin	Subunits	Glyco-protein	Isoelectric point	Genetic basis	Primary receptor	Mechanism of killing	Application
Bullera sinensis	?	?	?	?	Chromosomal	?	?	?
Candida krusei	?	?	?	3.6–3.8	?	?	?	?
Candida glabrata	?	?	+	?	Chromosomal	?	Plasma membrane damage	?
Cryptococcus humicola	?	< 1 kDa	?	?	Chromosomal	?	?	?
Debaryomyces hansenii	?	23 kDa	?	?	Chromosomal	β-(1→6)-Glucan	?	?
Hanseniaspora uvarum	?	18 kDa	–	3.7–3.9	dsRNA	β-(1→6)-Glucan	?	?
Kluyveromyces fragilis	K6	42 kDa	?	?	?	?	?	?
Kluyveromyces lactis	?	α(97 kDa) β(31 kDa) γ(28 kDa)	?	?	dsDNA (pGKL1)	Chitin < ß2 >	Inhibition of cell cycle, G1 arrest	Avoid aerobic deterioration of silage
Kluyveromyces waltii	?	> 10 kDa	?	?	?	?	?	Control of *S. pombe* in wine making
Pichia acaciae	?	α(110 kDa) β(39 kDa) γ(38 kDa)	?	?	dsDNA (pPac1–2)	Chitin	?	Cell cycle arrest in G1, chitinase activity
Pichia anomala	?	83 kDa	?	?	?	?	?	Control of filamentous fungi in wood
Pichia farinosa	SMKT	α (6.3 kDa) β (7.7 kDa)	+	?	Chromosomal	?	Increase membrane permeability to ions	?
Pichia fermentans	?	?	?	3.8–4.2	?	?	?	?
Pichia inositovora	?	> 100 kDa	?	?	dsDNA (pPin1–3)	?	?	?
Pichia kluyveri	?	19 kDa	+	4.3	Chromosomal	?	Formation of ion channel	?
Pichia membranifaciens	?	18 kDa[a]	–	3.9[a]	Chromosomal[a]	β-(1→6)-Glucan	Formation of ion channel[a]	?
Saccharomyces cerevisiae	K1	α(9.5 kDa) β (9 kDa)	–	4.5	M_1-dsRNA	β-(1→6)-Glucan	Formation of ion channels, activation of K^+ channel	Avoid undesired contaminants in wine, beer, sake, etc. Genetics
S. cerevisiae	K2	αβ(21.5 kDa)	+	4.2–4.3	M_2-dsRNA	β-(1→6)-Glucan	Increase membrane permeability to ions	Wine fermentations
S. cerevisiae	KT28	α(10 kDa) β(11 kDa)	+	4.4	M_{28}-dsRNA	Manno-proteins	Entering into cell by endocytosis and inhibition of cell cycle, G2 arrest	?
Schwanniomyces occidentalis	?	α(7.4 kDa) β(4.9 kDa)	–	?	Chromosomal	Manno-proteins	Plasma membrane damage	?
Tilletiopsis albenscens	?	10 kDa	?	?	Chromosomal	?	?	?
Williopsis mrakii	HM-1	10.7 kDa	?	?	Chromosomal	β-(1→6)-, β-(1→3)-glucan	Inhibition of β-(1→3)-glucan synthesis	Silage, yogurt, taxonomy of *Nocardia*, Control of *C. albicans*
Williopsis saturnus	HYI	9,543 Da	–	5.8	Chromosomal	?	?	?
Zygosacch. bailii	KT412	10 kDa	–	4.1	dsRNA	Manno-proteins	?	?

[a]Unpublished results

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Chapter 2

Isolation of yeast and molds from different types of cheese collected from local markets, Lincoln, Nebraska, USA in 2012-2013.

Abstract

Cheese serves as a complex microbial ecosystem. Properties of and interactions between microorganisms are responsible for the final character, quality and safety of cheese. Molds and yeasts play an important role in the quality and safety of cheese. Some yeasts may be a threat to food safety because they are opportunistic pathogens and can cause adverse conditions in humans. Similarly, some mold contamination and growth on cheese has been reported to produce mycotoxins and may present a potential health risk. There is an increasing concern regarding the safety of cheese, which is consumed by a majority of the population. In order to analyze the fungal composition and diversity in different types of cheeses, 48 different types of cheese were collected in 5 sampling periods. Molds and yeast were isolated in yeast extract glucose chloramphenicol medium (YEGC) and DNA was extracted. Polymerase chain reaction (PCR) was performed using the fungal-specific forward primer ITS1F and the universal eukaryotic reverse primer TW13. This amplified the ITS1, 5.8S, ITS2 and the 5' ~500 bp of the 28S regions of the nuclear ribosomal RNA genes. Samples were sequenced in both directions by Sanger sequencing and were identified using nucleotide BLAST against the NCBI non-redundant database. More than 50% of cheese found in the local market contained the yeast species *Debaryomyces hansenii,* with *Galactomyces geotrichum* being the second most abundant yeast species, while *Penicillium roqueforti* was the most frequently isolated mold in cheese. We

isolated some species capable of producing mycotoxins or causing human infections, but their frequency was low.

Introduction

Cheese is one of the most popular fermented milk products throughout the world (Fox & McSweeney, 2004; Lucey, 2008). It is a nutritious dairy food that contains high concentrations of essential nutrients such as fat, protein, vitamins, and minerals (Konuklar et al., 2004; O'Brien & O'Connor, 2004). Cheese making began about 8000 years ago (Fox & McSweeney, 2004) and has spread throughout the globe; currently there are more than 1000 cheese varieties (Irlinger & Mounier, 2009). In 2013 in the United States, total cheese production was 10.9 billion pounds - 2.8% above 2011 production (USDA, 2013).

Cheese production involves the transformation of milk into cheese via numerous biochemical reactions by microorganisms. Both starter bacteria and adventitious microorganisms are involved in cheese manufacturing and ripening. These also play a major role in obtaining the desired flavor, aroma, texture, color and nutritive elements in the cheese (Irlinger & Mounier, 2009; Ogier et al., 2002). So cheese contains a microbial ecosystem (Arfi et al., 2005) characterized by the presence of both starter cultures and environmentally obtained organisms. Fungi include starter species such as *Penicillium roqueforti* (blue cheeses), *P. camemberti* (mold-ripened cheeses such as Brie), and *Debaryomyces hansenii*, as well as non-starter yeasts and mold (NSYM) species (Mounier et al., 2006). Undesirable NSYM can be introduced into cheese by different sources of contamination such as air, farm workers, cheese makers, brine and cheese

making equipment (Borelli et al., 2006; Mounier et al., 2006; Pereira-Dias et al., 2000) and raw milk (Brooks et al., 2012). Some varieties of cheese manufactured in the United States use raw milk and age at temperatures not less than 1.7 C. (Brooks et al., 2012). Several studies have shown that some pathogens can survive this manufacturing and aging period (D'Amico et al., 2008; D'Amico et al., 2010). Similarly, some varieties of cheese provide an excellent substrate for yeast and molds to grow.

Yeasts present in various cheeses contribute to the taste and flavor; however, their presence is not always beneficial. Presence of different yeasts on the surface of the cheese can spoil and create an unpleasant smell or taste which affects the quality of cheese (Vasdinyei & Deák, 2003). Consumption of foods contaminated by certain yeasts can cause infections in immunocompromised individuals (Fleet, 2007). Such yeasts threaten food safety, given their association with opportunistic infections and other adverse conditions in humans (El-Sharoud et al., 2009; Fleet, 2007). Similarly to yeasts, some varieties of cheese contain molds as starter cultures which provide a characteristic appearance, consistency, flavor and prolonged self-life due to their protective effect against unwanted microorganisms (Haasum & Nielsen, 1998). However, adventitious molds contaminating and growing on cheese have been reported to produce mycotoxins and may present a potential health risk (Creppy, 2002; O'Brien et al., 2004; Sengun et al., 2008). Some cheese varieties are very susceptible to mold growth with a risk of mycotoxin production even in refrigerated conditions (Lund et al., 1995).

As part of our daily life, we purchase and consume many types of cheese which may contain yeasts and molds. The species and abundance of yeasts and molds may differ between different types of cheese; between cheeses of the same variety produced by

different manufacturers; and even between different batches of the same cheese from the same manufacturer (Lund et al., 1995). Raw milk cheese - of particular food safety concern - accounts for a very small part of the total domestic cheese market in the U.S. but the market for these cheeses continues to expand (West, 2008). Many of the fungal- and surface-ripened cheeses that have more recently become popular are considered to be at risk for pathogens (Brooks et al., 2012). Now, there is increasing concern regarding the safety of cheese (Brooks et al., 2012; Oliver, Jayarao, & Almeida, 2005). Yeasts and molds are rarely associated with outbreaks of foodborne infections, but serious attention should be focused on uncontrollable yeast and mold species. The aim of the present study was to analyze the fungal composition and diversity in different types of commercially available cheese.

Materials and Methods

Sampling of Cheese

Samples of 48 cheeses (differing in variety and/or manufacturer) were purchased from a local grocery store in Lincoln, Nebraska, USA from spring 2012 to May 2013. Samples were maintained at 4°C until analysis. The first three collections were taken in the spring, early summer and late summer of 2012. The fourth sampling was taken in April 2013 and the fifth in May 2013. Cheese sampling was based on the availability of cheese in the market at the time of sampling; not all cheeses were available at all samplings.

Isolation of Yeasts and molds

Sixty grams of each cheese was aseptically removed from the rind (3 samples-10 grams from each sample) and core (3 samples-10 grams from each sample). Each sample was

homogenized in 50 ml of sterile 1% peptone water using a Stomacher Lab Blender 400 (Seward Laboratory Systems, Davie, FL, USA) for five minutes at normal speed. Samples were serially diluted and plated on yeast extract-glucose chloramphenicol agar (YEGC agar: yeast extract 0.5%, glucose 2%, agar 1% and chloramphenicol 0.1%) and incubated for five days at 25 C. Individual colonies were selected based on morphology and color. The colonies were re-streaked on YEGC agar plates to ensure pure culture, incubated for four days at 25 C and maintained at 4 C until processing.

DNA isolation

Purified samples were grown in liquid YEGC media for 48 hours at 25 C and DNA was extracted from each isolate by following the modified method of Harju et al.,2004. Briefly, yeast colonies were picked into a microcentrifuge tube containing 200 µl of Harju buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl-pH8.0, 1mM EDTA). The tubes were placed at -80°C for 10 minutes and transferred immediately to a 95 C water bath for one minute. The freezing and heating process was repeated twice. Pestles were used to disrupt the yeast cells. Tubes were vortexed for 30 seconds. Two hundred µl of chloroform was added and the tubes were centrifuged at 20,000 x *g* for three minutes at room temperature. The upper aqueous layer was extracted and transferred to another microcentrifuge tube containing 400 µl ice-cold 100% ethanol, mixed by inverting gently and incubated at -20°C for 10 minutes. The tubes were again centrifuged at 20,000 rpm for five minutes at room temperature. The supernatant was removed and the pellet was washed with 500 µl 70% ethanol and centrifuged at 20,000 rpm for five minutes at room temperature. The supernatant was removed and the pellets were dried at room temperature and resuspended in 50 µl water.

Identification

Polymerase chain reaction (PCR) was performed using the fungal-specific primer ITS 1F (Gardes and Bruns 1993) and the universal eukaryotic primer TW13 (White et al. 1990) which amplified the ITS1, 5.8S, ITS2 and the 5' ~500 bp of the 28S regions of the nuclear ribosomal RNA genes. The amplification reaction was carried out in a BioRad thermal cycler with an initial denaturation step at 95°C for three minutes, followed by 30 cycles of 95°C for 20 seconds, 55 °C for 20 seconds, and 72°C for one minute and a final extension at 72°C for five minutes. Amplification products were analyzed on 0.7% agarose gel. PCR products were submitted for bidirectional Sanger sequencing to the Michigan State University Research Technology Support Facility. Samples were identified by sequence homology using nucleotide BLAST against the NCBI non-redundant database.

Results and Discussions

Collection of cheese samples

A total of 48 different cheeses were obtained from retail stores in Lincoln, Nebraska, USA. Sampling was dependent on cheese availability. Six cheeses were sampled four times, 9 were sampled three times, 10 were sampled twice, and. 23 cheeses were sampled only one time (**Appendix Tables A1-A3**)

Table 2-1: Mold and yeast species identified in cheese between Spring 2012 to May 2013.

	Sampling				
Cheese	**I**	**II**	**III**	**IV**	**V**
Very hard cheese					
Asiago (Bel Gioioso)	***	*Debaryomyces hansenii*	***	***	*D. hansenii*
Gruyere (Grand Cru Original)	***	*D. hansenii*	***	*D. hansenii* *Galactomyces geotrichum* *Penicillium commune* *Aspergillus tubingensis*	*D. hansenii*
Parmesan (Bel Gioioso)	***	*D. hansenii*	***	*D. hansenii* *Pueraria montana*	*D. hansenii* *Cladosporium perangustum*
Parmesan (Digiorno)	***	***	***	*P. solitum* **	**
Parmesan Reggiano	***	***	***	*D. hansenii* *Eurotium repens*	**
Zerto	***	*D. hansenii*	***	***	***
Hard cheese					
Cheddar (Black Creek Smooth and Creamy)	***	***	*D. hansenii*	***	***
Cheddar (Tickler Extra Mature)	***	**	*D. hansenii*	***	***
Cheddar, Goat	***	*G. geotrichum* *G. candidum* *Candida parapsilosis* *Candida sp.*	***	***	***
Jarlsberg	***	*D. hansenii*	*D. hansenii*	*D. hansenii*	*D. hansenii*

		G. geotrichum		*A. niger*	*P. solitum*
Romano (Bel Gioioso)	***	***	*D. hansenii*	***	***
Swiss (big eye)	***	*D. hansenii* *G. candidum*	*D. hansenii*	*D. hansenii* *C. parapsilosis*	*A. parvisclerotigenus*
Semi hard cheese					
Colby (Henning)	***	***	*D. hansenii*	***	***
Gouda (Heartland Creamery Legacy Creamy)	***	***	*D. hansenii* *C. sake*	***	***
Gouda (Landana 1000 Days)	***	***	*D. hansenii* *Candida sake*	***	***
Gouda, Smoked (Uniekaas)	***	*P. chrysogenum*	***	***	*D. hansenii* *A. niger* *Cladosporium sphaerospermum* **
Monterey Jack	***	*D. hansenii* *P. roqueforti* *Neospora caninum*	*D. hansenii* *P. roqueforti*	**	*D. hansenii*
Provolone	***	*D. hansenii*	***	*D. hansenii*	*D. hansenii*
Raclette (Grand Cru)	***	***	***	*D. hansenii*	***
Semi-Soft Cheese					
Fontina (Bel Gioioso)	***	*D. hansenii* *P. verrucosum*	***	**	*D. hansenii* *Taeniopygia guttata*
Haverti	***	*P. solitum*	***	***	***
Italian Bel Paese	***	*D. hansenii*	*D. hansenii*	*D. hansenii*	*D. hansenii*

				P. camemberti	*P. camemberti*
				P. gladioli	*P. solitum*
					P. commune
Muenster	***	*D. hansenii*	***	***	***
Wensleydale (Coombe Castle)	***	*D. hansenii*	***	*D. hansenii*	*D. hansenii*
				P. carneum	*Pichia kudriavzevii*
					P. solitum
					P. commune
Blue cheese					
Blue (Amablu)	***	***	***	*P. roqueforti*	*P. roqueforti*
				P. carneum	*P. carneum*
					D. hansenii
Blue (Mindaro)	***	*P. roqueforti*	***	*P. roqueforti*	*P. roqueforti*
		Ectomycorrhizal fungus		*Geomyces panorum*	*P. carneum*
		Phaeophleospora epicoccoides			
Blue (Roth Kase Minis)	*D. hansenii*	*P. roqueforti*	***	*D. hansenii*	*D. hansenii*
		P. paneum		*P. roqueforti*	*P. roqueforti*
					P. carneum
Blue Stilton	***	**	***	***	***
		D. hansenii			
		P. roqueforti			
		P. chrysogenum			
Gorgonzola	***	*P. roqueforti*	***	*P. roqueforti*	*P. roqueforti*
		P. paneum		*P. carneum*	*P. carneum*

Soft fresh cheese					
Cottage cheese	***	***	***	**	**
Cream cheese	***	***	***	**	**
Feta	***	*Kluyveromyces lactis*	***	**	*D. hansenii* *P. roqueforti* *P. carneum*
Mascarpone (Bel Gioioso)	***	**	***	*D. hansenii*	*P. carneum*
Mozzarella	***	*P. brevicompactum* *P. chrysogenum*	***	*P. roqueforti* *P. paneum* *P. carneum*	***
Ricotta	***	***	***	*D. hansenii* *P. roqueforti*	**
Wash rind Cheese					
Bavarian Red (Rougetta)	***	*D. hansenii*	*D. hansenii* *G. geotrichum*	*D. hansenii* *G. geotrichum* *Yarrowia lipolytica*	*D. hansenii* *G. geotrichum* *Yarrowia lipolytica* *P. roqueforti*
Non-classified cheese					
Dofino	***	***	*D. hansenii*	***	***
Mango Fire (Hennings)	***	***	*D. hansenii*	***	***
Queso Authentico	***	***	*D. hansenii*	***	***
Ranch Nuggets	***	***	*D. hansenii*	***	***
Sale Gran Queso	***	***	*D. hansenii*	***	***
Sartori Reserve	***	***	*D. hansenii*	***	***
Cream Gouda (Uniekaas)	***	***	*D. hansenii*	***	***
Wisconsin Roth Kase	***	***	*D. hansenii*	***	***

			P. roqueforti		
Monte Enebro	*G. geotrichum*	***	***	***	***
La tur Langa	*G. geotrichum*	***	***	***	***
Vermont Butter and Cheese Creamery	*D. hansenii*	***	***	***	***
Brie	***	**	***	***	***

I= Spring 2012, II= Early Summer 2012, III= Late Summer 2012, IV= April 2013, V= May 2013. **Cheese sampled, but no fungi isolated. ***Cheese not available for sampling at this time point.

Diversity of yeasts and molds in cheese

Yeasts and molds were identified to 28 species (**Table 2-1**). *Debaryomyces hansenii*, the most commonly isolated yeast, was isolated 63 times; the prevalent mold, *Penicillium roqueforti*, was isolated 20 times. Thirteen samples, representing 11 cheeses, did not yield yeasts or molds in culture.

Yeasts found in cheese

Nine different yeasts were isolated from 48 types of cheese in five samplings **(Table 2-2)**. Among these, *D. hansenii* was the most abundant species and was present in 50%, 55%, 95%, 54 % and 56 % of total cheese samples collected in spring 2012, early summer 2012, late summer 2012, April 2013 and May 2013 respectively. *D. hansenii* was detected in cheeses from all classifications: very hard, hard, semi hard, semi soft, soft fresh, blue and washed rind cheeses; and all sampling times. The prevalence of *D. hansenii* in cheese is due to its salt tolerance, ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat, capacity to grow at low temperatures and low water activity. *D. hansenii* can multiply in cheese, and assimilate lactate, citrate, lactose and galactose (Breuer & Harms, 2006; Welthagen & Viljoen, 1998). Besides, a synergistic effect between LAB and *D. hansenii* has been reported, which results in a long survival for the latter in cheese (Besancon et al., 1992; Breuer & Harms, 2006; Roostita & Fleet, 1996). Moreover, *D. hansenii* has been found to inhibit germination of undesired microorganisms by out-competing them for nutrients and producing antimicrobial metabolites (Breuer & Harms, 2006; Fatichenti, Bergere et al., 1983). Furthermore, *D. hansenii* can grow in the interior as well as on the surface of processed cheese, depending on the kind of cheese and the composition of the starter culture

Table 2-2: Number of yeasts isolated from cheeses between spring 2012 and May 2013

	Sampling				
Yeast	I (n=4)	II (n=27)	III (n=20)	IV (n=24)	V (n=25)
Debaryomyces hansenii	2	15	19	13	14
Galactomyces geotrichum	2	2	1	2	1
Galactomyces candidum	0	2	0	0	0
Candida parapsilosis	0	1	0	1	0
Candida sp	0	1	0	0	0
Candida sake	0	0	2	0	0
Pichia kudriavzevii	0	0	0	0	1
Kluyveromyces lactis	0	1	0	0	0
Yarrowia lipolytica	0	0	0	1	1

n=Number of cheese samples
I=Cheese sampling in Spring 2012, II=Sampling in Early Summer 2012, III= Sampling in Late Summer 2012, IV=Sampling in April 2013, V= Sampling in May 2013.

involved (Breuer & Harms, 2006; Fatichenti et al., 1983; G. Fleet, 1990).

Galactomyces geotrichum, the anamorph form of *Geotrichum candidum,* was the second most frequent yeast species, found in 50%, 7%, 5%, 8% and 4% of total cheese samples collected in spring 2012, early summer 2012, late summer 2012, April 2013 and May 2013 respectively; this was also isolated in each sampling period. Other yeasts isolated and identified in our study were *Candida parapsilosis, Galactomyces candidum, Candida sake, Pichia kudriavzevii, Kluyveromyces lactis and Yarrowia lipolytica.*

C. parapsilosis was isolated from cheddar (goat) and Swiss cheeses. *C. parapsilosis* is an emerging human pathogen that can cause invasive candidiasis (Trofa et al., 2008). These cheeses contained low levels of C. *parapsilosis*, which was isolated only one time from each cheese during our study. Two gouda cheese samples collected in late summer 2013 contained *Candida sake* which has been found in sake, fruit juice and is extensively used in food industries (Viñas et al., 1998). *C. sake* is a rare source of infection, but these

infections can be severe and include fungal endocarditis, peritonitis, oral candidiasis and bloodstream infections (Hoegl et al., 1998; Juneja et al., 2011). *Pichia kudriavzevii* was isolated from Wensleydale cheese in May 2013 but the population was low. It is reported as a common species in Graukase cheese and is also isolated from brines (Lavoie et al., 2012). It produces an antibiotic having excellent antibacterial activity against several human pathogens such as *Escherichia coli, Enterococcus faecalis, Klebsiella sp., Staphylococcus aureus, Pseudomonas aeruginosa* and *Pseudomonal alcaligenes* (Bajaj et al., 2013). *Kluyveromyces lactis* was isolated only one time, from a single sample of feta cheese (out of 3 samplings) and the population was low. *K. lactis* significantly influences the cheese ripening process and its presence in cheese contributes to longer survival of lactococci, increasing the concentration of some odorous compounds and change in cheese flavor; it is known for its ability to assimilate lactose (De Freitas et al., 2008).

Our study found *Y. lipolytica* only in Bavarian red, a washed rind cheese, from which it was isolated twice in April 2013 and May 2013. *Y. lipolytica* is a frequently occurring yeast species in blue cheeses and has an important role in production of important aroma compounds for cheese flavor (Gkatzionis et al., 2013); however, it is also regarded as one of the emerging opportunistic yeast pathogens although cases are rare (Jacques & Casaregola, 2008). A transient recurrent catheter-related fungemia has been attributed to *Y. lipolytica* in a leukemic patient (Chang et al., 2001).

Molds found in cheese

Nineteen different molds were isolated from 48 cheeses in five samplings **(Table 2-3)**. About 50% of molds were *Penicillium* species and 15% of them were *Aspergillus* species. Cheese is considered an adequate substrate for mold growth under suitable conditions of temperature and moisture. The incidence of molds in a wide variety of cheese indicates that *Penicillium* is the most common genus found in cheese. Distribution of these *Penicillium species* may be different in a manufacturing plant, ripening chambers and on the surface of cheeses.

Table 2-3: Number of molds isolated from cheeses between spring 2012 and May 2013

	Sampling			
Molds	II (n=27)	III (n=20)	IV (n=24)	V (n=25)
Aspergillus niger	0	0	1	1
Aspergillus parvisclerotigenus	0	0	0	1
Aspergillus tubingensis	0	0	1	0
Cladosporium perangustrum	0	0	0	1
Cladosporium sphaerospermum	0	0	0	1
Ectomycorrhizal fungi	2	0	1	0
Eurotium repens	0	0	1	0
Geomyces pannorum	0	0	1	0
Neospora canium	1	0	0	0
Penicillium brevicompactum	1	0	0	0
Penicillium camemberti	0	0	1	1
Penicillium carneum	0	0	4	6
Penicillium chrysogenum	2	0	0	0
Penicillium gladioli	0	0	1	0
Penicillium paneum	2	0	1	0
Penicillium roqueforti	5	2	6	6
Penicillium solitum	1	0	1	3
Penicillum commune	0	0	1	2
Phaeophleospora epicoccoides	1	0	0	0

n=Numbers of cheese samples
I=Cheese sampling in Spring 2012, II=Sampling in Early Summer 2012, III= Sampling in Late Summer 2012, IV=Sampling in April 2013, V= Sampling in May 2013.
Sampling I is missing in table due to absence of Molds species.

Among *Penicillium* species, *P. roqueforti* was present in 18%, 10%, 25% and 24% of cheese samples isolated in early summer 2012, late summer 2012, April 2013 and May 2013 respectively, but the abundance of *P. roqueforti* isolates differed among types of cheese (Table 3). All blue cheeses contained high populations of *P. roqueforti* (Table 1). Blue cheeses contain high populations of *P. roqueforti* due to the deliberate introduction of *P. roqueforti* as a starter culture in blue cheese (Houbraken et al., 2010), but it is impossible to confirm whether all *P. roqueforti* in cheese is part of the starter culture vs. natural contamination. Packaged cheese stored at refrigerated temperatures for a prolonged time creates a selective environment where *P. roqueforti* grows better than other *Penicillium* species (Kure et al., 2001). *P. roqueforti* is a major fungal contaminant in some cheeses in which it is not an intended additive (Tsai et al., 1988). This species can produce the secondary metabolites PR-toxin, roquefortine C, mycophenolic acid, and andrastin A (Bourdichon et al., 2012; Nielsen et al., 2005) but PR-toxin is unstable in cheese and the toxicity and role of the other potential mycotoxins in human health have not been conclusively demonstrated (Bourdichon et al., 2012). *P. roqueforti* was not frequently isolated in high populations from other types of cheeses. Low counts may indicate contamination of *P. roqueforti* from environment, workers, ripening chamber and other sources not from the culture.

Other *Penicillium* species which were isolated more than one time include *P. solitum, P. paneum, P. commune, P. carneum, P. camemberti* and *P. chrysogenum*. *P. solitum* was present in Parmesan, Jarlsberg and Wensleydale cheeses. This species is found in naturally fermented meats and does not produce any known mycotoxins (Bourdichon et al., 2012). *P. paneum* was isolated from gorgonzola and mozzarella cheese and is an

important fungal contaminant that is able to grow at low temperature, low pH, and high levels of carbon dioxide. *P. paneum* can produce mycotoxins, which may be harmful to humans (Chitarra et al., 2004). *P. camemberti,* which is used for production of white mold cheese, (Pitt et al.,1986) was isolated from Bel Paese (Italian) twice. *P.commune* was isolated from Gruyere, Wensleydale and Bel Paese (Italian) cheeses. Growth of *P. commune* on cheese results in discoloring of the surface and producing off flavors (Lund et al., 2003). Smoked Gouda and mozzarella contained *P. chrysogenum*, which is known for penicillin production. *P. carneum* was isolated from Wensleydale, mozzarella, four types of blue cheese (Amablu, Gorgonzola, Mindaro, and Roth Kase Minis), feta, and mascarpone. This result indicates that *P. carneum* is a more significant contaminant on these cheeses than the others. However, *P. carenum* is closely related to *P. roqueforti* and can be expected to inhabit similar niches. Some strains produce patulin and cyclopaldic acid (Boysen et al., 1996).

Nine mold species - *Aspergillus parvisclerotigenus, A. tubingensis, Phaeophleospora epicoccoides, Geomyces pannorum, Eurotium repens, Penicillium brevicompactum, P. gladioli, Cladosporium perangustum,* and *C. sphaerospermum* - were isolated only once. *A. parvisclerotigenus* which was isolated from a single sample of Swiss (big eye) cheese may produce aflatoxins (Rank et al., 2011). *A. tubingensis* belongs to the *Aspergillus* section *Nigri,* grows predominantly on dead plant materials and food, and was isolated from Gruyere cheese. Among four samplings, this species was isolated only once. *A. tubingensis* has been reported to cause severe invasive infections in immunocompromised hosts (Bathoorn et al., 2013). We isolated *P. epicoccoides* and *G. pannorum* from blue (Mindaro) cheese. *P. epicoccoides* is a plant pathogen and *G. pannorum* is a saprophytic

fungus frequently isolated from the soil and air samples. *G. pannorum* is a rarely reported animal and plant pathogen, causing occasional superficial infection of skin in humans (Gianni et al., 2003). *E. repens,* which was isolated only one time from Parmesan (Reggiano) cheese, has reported antibacterial, antifungal and antimalarial activities (Gao et al., 2012). *P. brevicompactum,* a common indoor mold which normally grows in building interiors in the presence of superfluous moisture, was present in one sample of mozzarella cheese (Scott et al., 2008). Presence of *P. brevicompactum* in mozzarella cheese may be due to contamination from an indoor environment. Similarly, *Cladosporium perangustrum* and *C. sphaerospermum* was isolated from Parmesan (Digiorno) and Gouda (smoked) respectively.

The variability in the microbiota composition can affect the quality and safety of cheese. In this study, yeasts and molds may have been unequally distributed on the surface and the inner part of cheese, therefore, sampling may have been biased although six samples were taken from each single cheese. Some species may have difficulties growing on the chosen media or were present at such low abundance that they were masked by the dominant species. Colonies were selected based on morphology, color, shape and size, so species with similar gross morphology may have been undersampled or undetected. It is difficult to know whether species are present from the starter culture (if used) or if they are natural contaminants. The populations of fungal species were not consistent (data not shown) in same and different cheeses. This may be due to contamination from different sources such as the environment, workers, ripening environment, handling sources and sampling bias. We did not consider the length of storage which can change the abundance

of fungal species. The longer the storage time, the higher chance of cheese contamination.

Human health and cheese quality is directly linked with the presence of molds and yeasts in cheese, so it is of interest to reduce contamination on cheese. It is important to examine the production line and identify possible points in the process where the cheese can be exposed to major mold and yeast contamination.

Appendix Table A1: Cheese sampled four times and isolated yeast and Molds

Cheeses sampled four times				
Jarlsberg	*Debaryomyces hansenii* *Galactomyces geotrichum*	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Aspergillus niger*	*Debaryomyces hansenii* *Penicillium solitum*
Swiss	*Debaryomyces hansenii* *Galactomyces candidum*	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Candida parapsilosis*	*Aspergillus parvisclerotigenus*
Monterey Jack	*Debaryomyces hansenii* *Penicillium roqueforti* *Neospora caninum*	*Debaryomyces hansenii* *Penicillium roqueforti*	**	*Debaryomyces hansenii*
Italian Bel Paese	*Debaryomyces hansenii*	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Penicillium camemberti* *Penicillium gladioli*	*Debaryomyces hansenii* *Penicillium camemberti* *Penicillium solitum* *Penicillium commune*
Bavarian Red	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Galactomyces geotrichum*	*Debaryomyces hansenii* *Galactomyces geotrichum* *Yarrowia lipolytica*	*Debaryomyces hansenii* *Galactomyces geotrichum* *Yarrowia lipolytica* *Penicillium roqueforti*
Blue (Roth Kase Minis)	*Debaryomyces hansenii*	*Penicillium roqueforti* *Penicillium paneum*	*Debaryomyces hansenii* *Penicillium roqueforti*	*Debaryomyces hansenii* *Penicillium roqueforti* *Penicillium carneum*

Appendix Table A2: Cheese sampled three times and isolated yeast and Molds

Cheeses sampled three times			
Gruyere	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Galactomyces geotrichum* *Penicillium commune* *Aspergillus tubingensis*	*Debaryomyces hansenii*
Parmesan (Bel Gioioso)	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Pueraria montana*	*Debaryomyces hansenii* *Cladosporium perangustum*
Provolone	*Debaryomyces hansenii*	*Debaryomyces hansenii*	*Debaryomyces hansenii*
Fontina	*Debaryomyces hansenii*	**	*Debaryomyces hansenii*
Wensleydale	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Penicillium carneum*	*Debaryomyces hansenii* *Pichia kudriavzevii* *Penicillium solitum* *Penicillium commune*
Feta	*Kluyveromyces lactis*	**	*Debaryomyces hansenii*
Mascarpone	**	*Debaryomyces hansenii*	*Penicillium carneum*
Blue (Mindoro)	*Penicillium roqueforti* *Phaeophleospora epicoccoides* **	*Penicillium roqueforti* *Geomyces panorum*	*Penicillium roqueforti* *Penicillium carneum*
Gorgonzola	*Penicillium roqueforti* *Penicillium paneum*	*Penicillium roqueforti* *Penicillium carneum*	*Penicillium roqueforti* *Penicillium carneum*

Appendix Table A3: Cheese sampled two times and isolated yeast and Molds

Cheese		
Asiago	*Debaryomyces hansenii*	*Debaryomyces hansenii*
Parmesan (Digiorno)	*Penicillium solitum*	**
	**	
Parmesan Reggiano	*Debaryomyces hansenii*	**
	Eurotium repens	
Cheddar	**	*Debaryomyces hansenii*
Gouda, smoked	*Penicillium chrysogenum*	*Debaryomyces hansenii*
		Aspergillus niger
		Cladosporium sphaerospermum
		**
Cottage Cheese	**	**
Cream Cheese	**	**
Mozzarella	*Penicillium brevicompactum*	*Penicillium roqueforti*
	Penicillium chrysogenum	*Penicillium paneum*
		Penicillium carneum
Ricotta	*Debaryomyces hansenii*	**
	Penicillium roqueforti	
Blue (Amahlu)	*Penicillium roqueforti*	*Penicillium roqueforti*
	Penicillium carneum	*Penicillium carneum*

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Chapter 3

The effect of *Debaryomyces hansenii* killer toxin against *Candida albicans* (SC5314) and *Candida tropicalis* (NRRL-10985)

Abstract

Candida yeasts are commensal members of the gastrointestinal, mucosal, oral and vaginal microbiota. However, when the host defense system and microbiota are disturbed, *Candida* can become pathogenic and cause severe candidiasis. Antifungal drugs targeted to treat candidiasis have been shown to result in treatment failures due to drug toxicity and/or development of resistance during long term antifungal therapy. *Debaryomyces hansenii* is the most common yeast species found in cheeses and is among the most common viable yeasts found in foods. Some strains of *D. hansenii* produce killer toxins - toxic proteins or glycoproteins which can kill sensitive yeast species. Therefore, we investigated whether *D. hansenii* isolated from different cheese samples had an inhibitory role on *Candida* species. Forty two *D. hansenii* isolates were collected from different types of cheese and killer toxin activity against *Candida albicans* and *Candida tropicalis* was screened by the streak-plate agar diffusion bioassay. Killer activity among *D. hansenii* strains at different pH values (4.5, 5.0, 5.5, 6.0) and temperatures (20 C, 25 C, 30 C and 35 C) was quantified by agar diffusion well bioassay; the effect of *D. hansenii* killer toxin on *C. albicans* and *C. tropicalis* growth kinetics was also studied. Twenty three strains (54%) of *D. hansenii* demonstrated killer activity against *C. albicans* and *C. tropicalis* and killer toxin activity differed among the *D. hansenii* strains. *D. hansenii* killer toxin was active against *C. albicans* up to pH 5.5 but

against *C. tropicalis* to pH 6.0. Killer activity was higher at low temperature and low pH. Killer toxin activity was detected up to 35 C against *C. albicans;* for *C. tropicalis*, lower temperatures were required to observe a killer effect. *C. albicans* was more sensitive to killer toxin than *C. tropicalis* at high temperature while *C. tropicalis* was more sensitive than *C. albicans* at low temperature. The results confirmed that same killer toxin from *D. hansenii* can act differently in different species, temperature and pH conditions; strains such as Dhans-237, which have activity at higher temperature, may have medical application.

Introduction

Candida species form part of the normal microbiota of the human mucosal oral cavity, vagina, and gastrointestinal tract (Moran et al., 2012; Sardi et al., 2013; Williams et al., 2013). Several species, including *Candida albicans, C. dublinensis, C. glabrata, C. guilliermondii, C. lusitaniae, C. parapsilosis* and *C. tropicalis,* can be found as part of the normal human commensal flora, especially in all sections of the gastrointestinal tract (Hube, 2004; Netea et al., 2008; Schulze & Sonnenborn, 2009). However, in response to change or disturbance in the host defense systems in the gut, including the intestinal microbiota, gut-associated immune system and the mucosal barrier, *Candida* species can convert from harmless commensals into pathogens (Liu, 2002; Netea et al., 2008; Walker et al., 2009). These opportunistic pathogens produce a spectrum of disease, ranging from superficial mucosal infection to systemic candidiasis (Moran et al., 2012; Vargas & Joly, 2002; Walker et al., 2009). Most of the infections appear to be endogenously acquired from the normal commensal *Candida* species in the intestinal tract (Moran et al., 2012;

Perlroth et al., 2007). In recent years, the incidence of *Candida* infections has increased dramatically due to the rise in the number of immunocompromised patients (Gozalbo et al., 2004). Also, there has been a marked increase in the incidence of treatment failures in candidiasis patients receiving long-term antifungal therapy (Mishra et al., 2007).

Some yeast can produce toxic proteins or glycoproteins called killer toxins or mycocins, which can kill sensitive yeast isolates (Schmitt & Breinig, 2002). Killer toxin activity has been reported from more than 100 yeast species belonging to more than 20 genera, and the killer character does not appear uniformly among these strains, nor can it be linked to the sources of their isolation (Buzzini & Martini, 2001; Young & Yagiu, 1978). *Debaryomyces hansenii* is the most common yeast species found in cheese due to its ability to grow in the presence of high salt (Prista et al., 2005), low pH, low water activity (Capece & Romano, 2009) and its ability to metabolize lactic and citric acids (Ferreira & Viljoen, 2003). *D. hansenii* can produce killer toxins (Buzzini & Martini, 2001; Santos, et al., 2002); it does not represent a human pathogen but has been detected in the human GI tract (Desnos-Ollivier et al., 2008).

Preliminary pyrosequencing data (Hallen-Adams, unpublished) of gut-associated fungi from 45 healthy humans detected *D. hansenii* in 16% of all fecal samples, while opportunistic pathogenic *Candida* species were detected in 75%. The presence of significant counts of *D. hansenii* (>100 sequencing hits) correlated with fewer hits to pathogenic *Candida* species than average and, in five of these humans, *Candida* counts were zero.

We consume many types of cheese which contain *D. hansenii* as the dominant species (Banjara, Chapter 2, this thesis). The preliminary sequencing data and reports of killer toxin production by *D. hansenii* suggest killer toxin production by foodborne strains may play a role in reducing the natural gut *Candida* population. This leads us to investigate whether *D. hansenii* isolated from different cheese samples has an inhibitory role on *Candida* species or not.

The objectives of our study were to:

1) Evaluate killer toxin activity of multiple food-derived *D. hansenii* strains against two opportunistic pathogenic *Candida* species, *C. albicans* and *C. tropicalis*.
2) Quantify the killer activity of toxin produced by *D. hansenii* strains against *C. albicans* and *C. tropicalis* at different temperature and pH.
3) Examine the effect of killer toxin produced by *D. hansenii* on growth kinetics in *C. albicans* and *C. tropicalis.*

Materials and Methods

Isolates used

Forty two *D. hansenii* strains were isolated from different cheeses purchased from local grocery stores in Lincoln, NE, USA. The complete list of *D. hansenii* strains and sources of isolation is given in Table 3-1.

Table 3-1: List of *Debaryomyces hansenii* strains and source of isolation

Cheese	***D. hansenii* strain**	**Cheese**	***D.hansenii* Strain**
Belgioloso Romano	Dhans-3	Queso Authentico	Dhans-68
Queso Sale Gran	Dhans-7	Hennings Colby	Dhans-72
Queso Authentico	Dhans-10	Italian Belpase	Dhans-75
Beelgioloso Romano	Dhans-21	Cheddar (Black Creek Smooth and Creamy)	Dhans-76
Cream Gouda(Uniekaas)	Dhans-23	Hennings Colby	Dhans-79
Legacy cheese	Dhans-33	Mango Fire(Hennings)	Dhans-80
Italian Belpase	Dhans-34	Ranch Nuggets	Dhans-103
Italian Belpase	Dhans-43	Jarlsberg (Norwegian)	Dhans-107
Mango Fire (Hennings)	Dhans-44	Landana 1000 days (Gouda)	Dhans-111
Belgioloso Romano	Dhans-45	Landana 1000 days (Gouda)	Dhans-121
Wisconsin Roth Kase	Dhans-46	Bavarian Red (Rougetta)	Dhans-201
Mango Fire (Hennings)	Dhans-48	Italian Bel Paese (Galbani)	Dhans-220
Legacy cheese	Dhans-51	Italian Bel Paese	Dhans-237
Bavarian Red, Rougette	Dhans-53	Parmesan (Reggiano)	Dhans-242
Ranch Nuggets	Dhans-55	Raclette(Grand Cru)	Dhans-246
Hennings Colby	Dhans-56	Mascarpone (Belgioioso)	Dhans-254
Hennings Colby	Dhans-60	Provolone cheese (Dilusso's Wisconsin)	Dhans-255
Gouda (Uniekaas)	Dhans-61	Ricotta	Dhans-262
Jarlaberg (Norwegian)	Dhans-63	Wensleydale cheese (Coombe castle)	Dhans-265
Landana 1000 days (Gouda)	Dhans-65	Blue cheese (Roth Kase Minis)	Dhans-274
Gouda (Uniekaas)	Dhans-66	Blue cheese (Roth Kase Minis)	Dhans-276

Candida albicans strain SC5314 was obtained as gift from Dr. Nickerson's lab,

University of Nebraska-Lincoln, and *Candida tropicalis* NRRL 10985 was obtained from

the Agriculture Research Service (ARS) Culture Collection, National Center for Agricultural Utilization Research, U.S Department of Agriculture, Peoria, Illinois.

1. Screening killer toxin activity against *C. albicans* and *C. tropicalis*

The killer toxin activity against *C. albicans* and *C. tropicalis* was evaluated by streak-plate agar diffusion bioassay **Fig 3-1** (Hodgson et al., 1995; Rosini, 1983). YEPD-methylene blue agar (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar, 0.003% methylene blue and 10 mM sodium citrate) was prepared to varying pH values (4.5, 5.5, 6.5 and 7.0). Citrate is an appropriate buffer for these pH values because citric acid has pKa values 3.13, 4.76 and 6.40. Hydrochloric acid was used to adjust the pH. Media was autoclaved and was cooled to 45 C and seeded with *C. albicans* or *C. tropicalis* at a density of 10^5 cells ml^{-1}, and 15 ml was poured into each plate. A colony of each *D. hansenii* strain was streaked on the agar surface whereas they all look as if they are circular and plates were incubated at 20 C, 25 C, 30 C and 35 C for 3-7 days. The experiment was performed in triplicate. Development of a clear inhibition zone around the *D. hansenii* colony was considered as a positive indication of killer toxin activity, whereas absence of a clear or blue zone indicated a lack of killer toxin activity (Hernandez et al., 2008).

2. Quantify the *D. hansenii* killer toxin activity against *C. albicans* and *C. tropicalis* at different pH and temperature

Crude toxin preparation

Three *D. hansenii* strains (Dhans-274, Dhans-237 and Dhans-65) with killer activity and one killer-negative strain (Dhans-3) were selected to quantify the killer toxin activity

against *C. albicans* and *C. tropicalis.* The selected *D. hansenii* strains with 10^6 cells^{-ml} were grown in 200 ml liquid YEPD media at pH 4.5 for 72 hours at 25 C in a 250-ml Erlenmeyer flask. The cells were pelleted by centrifugation (Beckman, USA) at 2750 x *g* for 10 minutes at 4 C and discarded, and the culture supernatant was filtered through a 0.45 µm pore diameter membrane. The clarified extracts were lyophilized (Labconco, USA) for 3 days and 5 grams of each dried sample was reconstituted in 15 ml of sterile YEPD media (Hodgson, Walker, & Button, 1994) to yield crude toxin. Two hundred ml of uninoculated YEPD media was freeze-dried and 5 grams of dry sample was reconstituted as above and used as a control. The crude toxin dry sample contains salts and other media components and variable, very low level of protein. Dhans-3, which lacked killer activity against *C. albicans* and *C. tropicalis,* was used as a negative control.

Agar diffusion well bioassay

YEPD-methylene blue medium was prepared to varying pH values (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) and autoclaved. Media was cooled to 45 C and seeded with *C. albicans* or *C. tropicalis* at a density 10^5 cells ml^{-1} and 15 ml were dispensed into each plate. Wells were cut in the solidified agar using a sterile borer (7 mm diameter) and agar plugs removed with a sterile scalpel. Crude toxin samples (100 µl) were pipetted into the wells and the plates were incubated at 20, 25, 30 and 35 C for 2 days. A clear zone of inhibition around the wells was indicative of killer activity, and this clear zone was measured by using software Microplate manager-6 (Bio Rad Lab, Inc., USA) which is more accurate and does not take more time. The killing activity of each sample was measured and defined as the mean of inhibition of three replicate wells (Hodgson et al., 1995). The larger the zone of inhibition, the higher was the killer toxin activity and vice versa.

Statistical analysis.

Significant differences between the treatments were determined using two-way ANOVA. Comparisons were done using using Tukey's multiple comparison tests. Differences between treatments were considered significant when P-values were less than 0.05. GraphPad Prism 6 (GraphPad Software, Inc, 2013) was used to perform statistical tests and to generate graphical images.

3. Effect of *D. hansenii* killer toxin on growth kinetics in *C. albicans* and *C. tropicalis*

Liquid YEPD medium at pH 4.5 was seeded with 10^5 cells ml^{-1} of *C. albicans* or *C. tropicalis* and 200 µl aliquots were dispensed in the wells of a microtitre plate. Uninoculated medium was used as a control. Fifty µl of Dhans-237 crude toxin, Dhans-3 culture filtrate or uninoculated control was mixed in each well and incubated at 25 °C, 30 °C and 35 °C. The cell growth of *C. albicans* or *C. tropicalis* was monitored using a microplate reader (Bio Rad, Japan) (595 nm) over a 24 hour period. The plate was mixed prior to growth measurements. The experiment was performed in triplicate.

Results and Discussion

1. Screening of killer toxin activity

A total of 42 *D. hansenii* strains isolated from different types of cheeses (**Table 3-1**) were used to screen killer toxin activity against *C. albicans* and *C. tropicalis*. At low temperature (20 C) with low pH (4.5), 22 strains (52%) showed killer toxin activity against *C. albicans* and 23 strains (54%) had killer toxin activity against *C. tropicalis* (Table 3-2 and Table 3-3). At higher temperature with high pH, fewer strains

demonstrated killer activity). Above 30 C, we did not observe any killer activity against either *Candida* species.

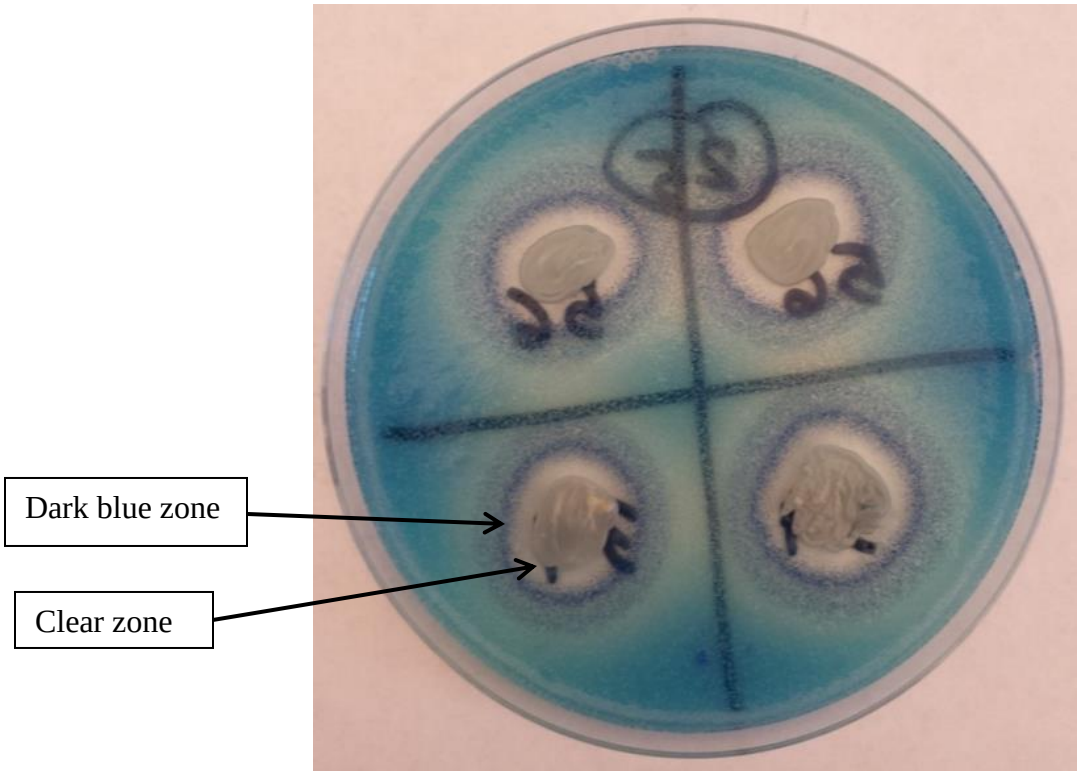

Figure 3-1: Streak plate assay. A YEPD methylene blue agar plate is seeded with susceptible *Candida albicans*, then streaked with *Debaryomyces hansenii* testor strains. The *D. hansenii* strains shown are killer, as indicated by the clear zone immediately surrounding the *D. hansenii*, and the dark blue zones of killed *Candida* cells.

Table 3-2. Killer toxin activity of *D. hansenii* strains isolated from cheese against *C. albicans* at varying temperatures and pH values.

Strain	20 C pH 4.5	20 C pH 5.5	25 C pH 4.5	25 C pH 5.5	30 C pH 4.5
Dhans-3	-	-	-	-	-
Dhans-7	-	-	-	-	-
Dhans-10	K	K	K	K	-
Dhans-21	K	S	-	-	-
Dhans-23	-	-	-	-	-
Dhans-33	-	-	-	-	
Dhans-34	K	K	K	K	K
Dhans-43	K	K	K	K	-
Dhans-44	-	-	-	-	-
Dhans-45	K	K	K	K	-
Dhans-46	K	K	K	K	-
Dhans-48	-	-	-	-	-
Dhans-51	K	S	K	S	-
Dhans-53	-	-	-	-	-
Dhans-55	-	-	-	-	-
Dhans-56	K	S	K	K	-
Dhans-60	-	-	-	-	-
Dhans-61	-	-	-	-	-
Dhans-63	-	-	-	-	-
Dhans-65	K	K	K	K	-
Dhans-66	-	-	-	-	-
Dhans-68	-	-	-	-	-
Dhans-72	-	-	-	-	-
Dhans-75	-	-	-	-	-
Dhans-76	-	-	-	-	-
Dhans-79	-	-	K	K	-
Dhans-80	-	-	-	-	-
Dhans-103	K	K	K	K	-
Dhans-107	S	S	-	-	-
Dhans-111	-	-	-	-	-
Dhans-121	K	K	K	K	-
Dhans-201	K	K	K	K	K
Dhans-220	K	K	K	K	K
Dhans-237	K	K	K	K	K

Dhans-242	K	K	K	K	K
Dhans-246	K	S	S	S	K
Dhans-254	K	K	K	K	-
Dhans-255	K	K	K	K	K
Dhans-262	K	K	K	K	-
Dhans-265	K	K	K	K	K
Dhans-274	K	K	K	K	K
Dhans-276	K	S	K	K	-

K = kill, S = sensitive, - = does not kill
All temperatures were tested at pH 5.5, 5.5, 6.5 and 7.0. No anti-*C. albicans* activity was observed at pH 6.5 or pH 7.0 at any temperature; additionally, no activity was observed at pH 5.5 at 30 C.

Table 3-3. Killer toxin activity of *D. hansenii* strains isolated from cheese against *C. tropicalis* at varying temperatures and pH values.

Strain	20 C pH 4.5	20 C pH 5.5	25 C pH 4.5	25 C pH 5.5	30 C pH 4.5
Dhans-3	-	-	-	-	-
Dhans-7	-	-	-	-	-
Dhans-10	K	K	K	K	K
Dhans-21	K	-	-	-	-
Dhans-23	-	-	-	-	-
Dhans-33	-	-	-	-	-
Dhans-34	K	K	K	K	K
Dhans-43	K	K	K	K	-
Dhans-44	-	-	-	-	-
Dhans-45	K	K	K	K	K
Dhans-46	K	K	K	K	-
Dhans-48	-	-	-	-	-
Dhans-51	K	K	K	K	-
Dhans-53	-	-	-	-	-
Dhans-55	-	-	-	-	-
Dhans-56	K	K	K	K	-
Dhans-60	-	-	-	-	-
Dhans-61	-	-	-	-	-
Dhans-63	-	-	-	-	-

Dhans-65	K	K	K	K	K
Dhans-66	-	-	-	-	-
Dhans-68	-	-	-	-	-
Dhans-72	-	-	-	-	-
Dhans-75	-	-	-	-	-
Dhans-76	-	-	-	-	-
Dhans-79	-	-	-	-	-
Dhans-80	-	-	-	-	-
Dhans-103	K	K	K	K	-
Dhans-107	K	K	S	S	-
Dhans-111	S	-	-	-	-
Dhans-121	K	K	K	K	-
Dhans-201	K	K	K	K	K
Dhans-220	K	K	K	K	K
Dhans-237	K	K	K	K	K
Dhans-242	K	K	K	K	-
Dhans-246	K	K	K	K	K
Dhans-254	K	K	K	K	-
Dhans-255	K	K	K	K	K
Dhans-262	K	K	K	K	-
Dhans-265	K	K	K	K	K
Dhans-274	K	K	K	K	K
Dhans-276	K	K	K	K	S

K = kill, S = sensitive, - = does not kill
All temperatures were tested at pH 5.5, 5.5, 6.5 and 7.0. No anti-*C. tropicalis* activity was observed at pH 6.5 or pH 7.0 at any temperature; additionally, no activity was observed at pH 5.5 at 30 C.

Nineteen *D. hansenii* strains displayed no killer activity against either *C. albicans* SC5314 or *C. tropicalis* NRRL-10985 under any conditions tested. However, these strains may have killer activity against other yeast strains or species, or under different conditions (i.e., elevated NaCl) because not all strains of the same species produce the same patterns of activity (Hodgson et al., 1995). One yeast species can produce multiple

toxins, differing in activity at different pH and temperature (Hernandez et al., 2008; Michaláková et al., 1993). So we screened killer activity at several physiologically meaningful pHs and temperatures. We did not observe *D. hansenii* strains which had killer toxin activity against *C. albicans* but not *C. tropicalis* or vice versa.

2. Quantify the *D. hansenii* killer toxin activity against *C. albicans* and *C. tropicalis* at different pH and temperature

The killer activities of *D. hansenii* toxin against *C. albicans* and *C. tropicalis* were quantified at different pH (4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) and various temperatures (20 C, 25 C, 30 C and 35 C). The results in Table 3-4 and Table 3-5 show that numerous variables influence the performance of killer toxin against susceptible yeast cells. The impact of crude toxin on *Candida* was dependent on the pH and temperature at which the assay was conducted; the *D. hansenii* strain that produced the toxin; and the sensitive *Candida* isolate. Activity of Dhans-237 crude toxin differed significantly from that of Dhans-65 against *C. albicans* in all pH and temperatures except pH 5.0 at 20 C and 35 C. Similarly, killer toxin activity of Dhans-237 differed significantly from that of Dhans-65 against *C. tropicalis* at all pH and temperatures except at pH 4.5 at 30 C and pH 5 at 25 C. Dhans-237 killer toxin consistently displayed the greatest activity of the three tested killer strains, and Dhans-65 displayed the least. Culture filtrate from killer negative *D. hansenii* strain Dhans-3 and the negative control had no significant effect on *Candida* growth.

Table 3-4: Effect of temperature on killer toxin activity against *Candida albicans* at different pH

Strains	pH	Zone of inhibition (mm)* Temperature (˚C)				pH	Zone of inhibition (mm)* Temperature (˚C)				pH	Zone of inhibition (mm)* Temperature (˚C)			
		20	25	30	35		20	25	30	35		20	25	30	35
Dhans-274	4.5	8^{a1}	7.72^{a1}	4.83^{b1}	3.93^{b}	5.0	4.74^{a1}	3.74^{b1}	3.30^{c1}	2.94^{c1}	5.5	3.59^{a1}	3.15^{a1}	2.27^{b1}	0
Dhans-237	4.5	8.13^{a1}	8.70^{a1}	5.79^{b2}	4.30^{c1}	5.0	4.84^{a1}	4.35^{a2}	3.81^{c2}	3.03^{c1}	5.5	3.89^{a2}	3.53^{a2}	3.38^{a2}	0
Dhans-65	4.5	7.15^{a2}	7.44^{a2}	4.59^{b1}	2.43^{c2}	5.0	4.50^{a1}	3.54^{b1}	2.60^{c1}	2.54^{c1}	5.5	2.76^{a1}	2.43^{a3}	2.27^{a1}	0
Dhans-3	4.5	0	0	0	0	5.0	0	0	0	0	5.5	0	0	0	0
Control	4.5	0	0	0	0	5.0	0	0	0	0	5.5	0	0	0	0
SE	0.37					0.23					0.14				

Table 3-5: Effect of temperature on killer toxin activity against *Candida tropicalis* at different pH

Strains	pH	Zone of inhibition * Temperature (˚C)				pH	Zone of inhibition* Temperature(˚C)			
		20	25	30	35		20	25	30	35
Dhans-274	4.5	15.09^{a1}	13.80^{b1}	3.27^{c1}	0	5.0	14.34^{a1}	7.76^{b1}	0	0
Dhans-237	4.5	15.67^{a1}	13.86^{b1}	3.20^{c1}	0	5.0	15.62^{a2}	8.59^{b1}	0	0
Dhans-65	4.5	14.59^{a2}	13.45^{b2}	2.22^{c1}	0	5.0	14.52^{a1}	7.79^{b1}	0	0
Dhans-3	4.5	0	0	0	0	5.0	0	0	0	0
Control	4.5	0	0	0	0	5.0	0	0	0	0
SE	0.31					0.35				

Strains	pH	Zone of inhibition * Temperature(˚C)				pH	Zone of inhibition* Temperature(˚C)			
		20	25	30	35		20	25	30	35
Dhans-274	5.5	10.34^{a1}	6.55^{b1}	0	0	6.0	4.92^{a1}	2.29^{b1}	0	0
Dhans-237	5.5	9.38^{a2}	6.84^{b1}	0	0	6.0	4.79^{a1}	3.09^{b2}	0	0
Dhans-65	5.5	8.62^{a3}	5.09^{b2}	0	0	6.0	3.25^{a2}	2.10^{b1}	0	0
Dhans-3	5.5	0	0	0	0	6.0	0	0	0	0
Control	5.5	0	0	0	0	6.0	0	0	0	0
SE	0.19					0.23				

***** Zone of inhibition= diameter of the clear zone (mm); Data are given as means with standard error (SE), n=3
Different superscript letters signify statistical different in killer toxin activity among different temperature and different numbers signify killer toxin activity different among different strain

Killer toxin activity against *C. albicans* and *C. tropicalis* varied with temperatures and pH (Table 3-4 and Table 3-5). Killer activity was higher in low temperature at low pH but as temperature and pH increased, killer activity decreased. Killer activity of the three tested *D. hansenii* strains against *C. albicans* was detected in all temperatures at pH 4.5, 5.0 and 5.5, but activity was absent at pH 5.5 at 35 C. Each strain differed significantly in killer toxin activity between 20 and 35 C. Killer activity was highest at pH 4.5 with low temperatures (20 and 25 C) and lowest at pH 5.5 and 30 °C. Killer activity against *C. tropicalis* was detected at all tested pH values at low temperatures (20 and 25 °C) while activity was absent at 35 C. In each strain, there is significant difference in killer toxin activity between 20 C, 25 C, and 30 C at pH 4.5, 5.0, 5.5 and 6.0. At low pH (4.5) with low temperature (20 C) the highest killer activity was displayed, while pH 6.0 with 25 C induced the lowest detectable killer activity.

The stability of all killer toxins is strongly dependent on pH and temperature (Hernandez et al., 2008; Marquina, Barroso, Santos, & Peinado, 2001). We found that killer toxin of the strains Dhans-274, Dhans-237 and Dhans-65 had toxin activity against *C. albicans* up to pH 5.5 and against *C. tropicalis* up to pH 6.0. Most studies performed with killer toxin activity found that killer toxins are generally stable only over a narrow pH ranges and each toxin has a defined optimal pH for killer activity against the sensitive yeast species (Chen et al., 2000; Soares & Sato, 2000). A previous report by Marquina et al., (2001) stated that killer toxin from *D. hansenii* isolated from olive brines had an optimal stability and activity against *Candida boidinii* (IGC3430) from pH 4.5 to pH 5.1. Buzzini and colleagues (Buzzini et al., 2004) report that a killer toxin produced by the tropical yeast

Williopsis saturnus is active over a broad range of pH (4.5-8.0). Our result differed slightly with the observations of Marquina and colleagues (2001), and that might be due to different *D. hansenii* strains, different sources for the strains, and/or different sensitive species.

Similarly, we found killer toxin activity against *C. albicans* up to 35 C and against *C. tropicalis* up to 30 C. At the lowest tested temperature (20 C), killer activity was high but with increase in temperature, killer activity against both *Candida* species decreased. That might be due to inactivation of *D. hansenii* killer toxin at higher temperatures (Hernandez et al., 2008; Marquina et al., 2001).

Sensitivity of *C. albicans* and *C. tropicalis* significantly differed at multiple pH values (4.5, 5.0 and 5.5) and temperatures (20, 25, 30 and 35 C). *C. tropicalis* was more sensitive to the *D. hansenii* killer toxins than *C. albicans* at lower temperatures, while *C. albicans* was more sensitive than *C. tropicalis* to killer toxins at high temperature. This result indicates it is possible that same killer toxin displays a different spectrum of activity against different species (Hernandez et al., 2008).

3. Effect of *D. hansenii* killer toxin on growth kinetics in *C. albicans* and *C. tropicalis*

This experiment was conducted at pH 4.5 because most killer strains produce toxins which are active at this pH (Middelbeek, Hermans, Stumm, & Muytjens, 1980; supported by our own results). Crude toxin from Dhans-237 was selected for this study due to its displaying the highest killer toxin activity against *C. albicans* and *C. tropicalis* (Tables 3-4 and 3-5). The effect of Dhans-237 killer toxin on the growth kinetics of *C. albicans* and *C. tropicalis* was monitored by smicroplate reader (OD_{595}) over 24 hours (Figure 3-1).

When *Candida* cells were incubated with toxin there was a reduction in the turbidity of the culture compared with control treatments. Lag phase was observed 15 hours at 25 C, 15 hours at 30 C and 11 hours at 35 C after addition of Dhans-237 toxin or control in the growing media containing *C. albicans*. Lag phase occurred earlier for *C. tropicalis*: in 10 hours at 25 C, 8 hours at 30 C and 5 hours at 35 C. Following lag phase, killer toxin from Dhans-237 strain exhibited greater growth inhibition on *C. albicans* and *C. tropicalis* than Dhans-3 and control treatments. *C. albicans* and *C. tropicalis* growth with Dhans-3 and control treatments showed the same, uninhibited growth pattern at all three temperatures (25, 30 and 35 C). Dhans-237 had a greater inhibitory effect on *C. albicans* and *C. tropicalis* growth at lower temperatures and a lower effect at higher temperatures. At the highest tested temperature, 35 C, *C. albicans* growth in the presence of Dhans-237 killer toxin remained low compared to growth with Dhans-3 and control treatment. In contrast, killer toxin from Dhans-237 showed no effect on *C. tropicalis* growth at 35 C.

The mode of action for *D. hansenii* killer toxin has not yet been fully characterized. Binding of toxin to cell wall is commonly the first event in the action of killer toxin against sensitive cells. Numbers of toxin receptors are available on cell wall of yeast (Hutchins & Bussey, 1983; Santos et al., 2000; Schmitt & Radler, 1988). On incubation of toxin with sensitive yeast cells, toxin molecules immediately bind to receptors of sensitive cells (Hodgson et al., 1995; Santos & Marquina, 2004). Sensitive cells die due to various causes, including formation of ion channels, inhibition of cell division at the early G2 stage, inhibition of DNA synthesis and β-1, 3-glucan synthesis. β-Glucans are the major components of the yeast cell wall and have been proposed as primary receptors of killer toxins. Santos et al. (2002) report that the killer toxin produced by *D. hansenii*

strain CYC 1021 is primarily adsorbed by (1→6)-β-D-glucan but the detail of the killing mechanism remains unknown. It is possible that different availability of toxin receptors available on *C. albicans* and *C. tropicalis* cell walls may lead to greater or less killing of the two species. In initial hours Dhans-237 toxin showed high stability and activity at all temperatures; however, as the time of incubation increased, activity decreased. That might be due to growth of *C. albicans* and *C. tropicalis* exceeding available toxin molecules, or deactivation of toxin with temperature and time of incubation (Bajaj et al., 2013). Other possibilities may be inactivation of killer toxin due to protease derived from the yeast cells (Woods & Bevan, 1968). So decreased growth inhibition of *C. albicans* and *C. tropicalis* by killer toxin at high temperature might be due to decreased toxin binding at high temperatures and present of protease produced by yeast cells.

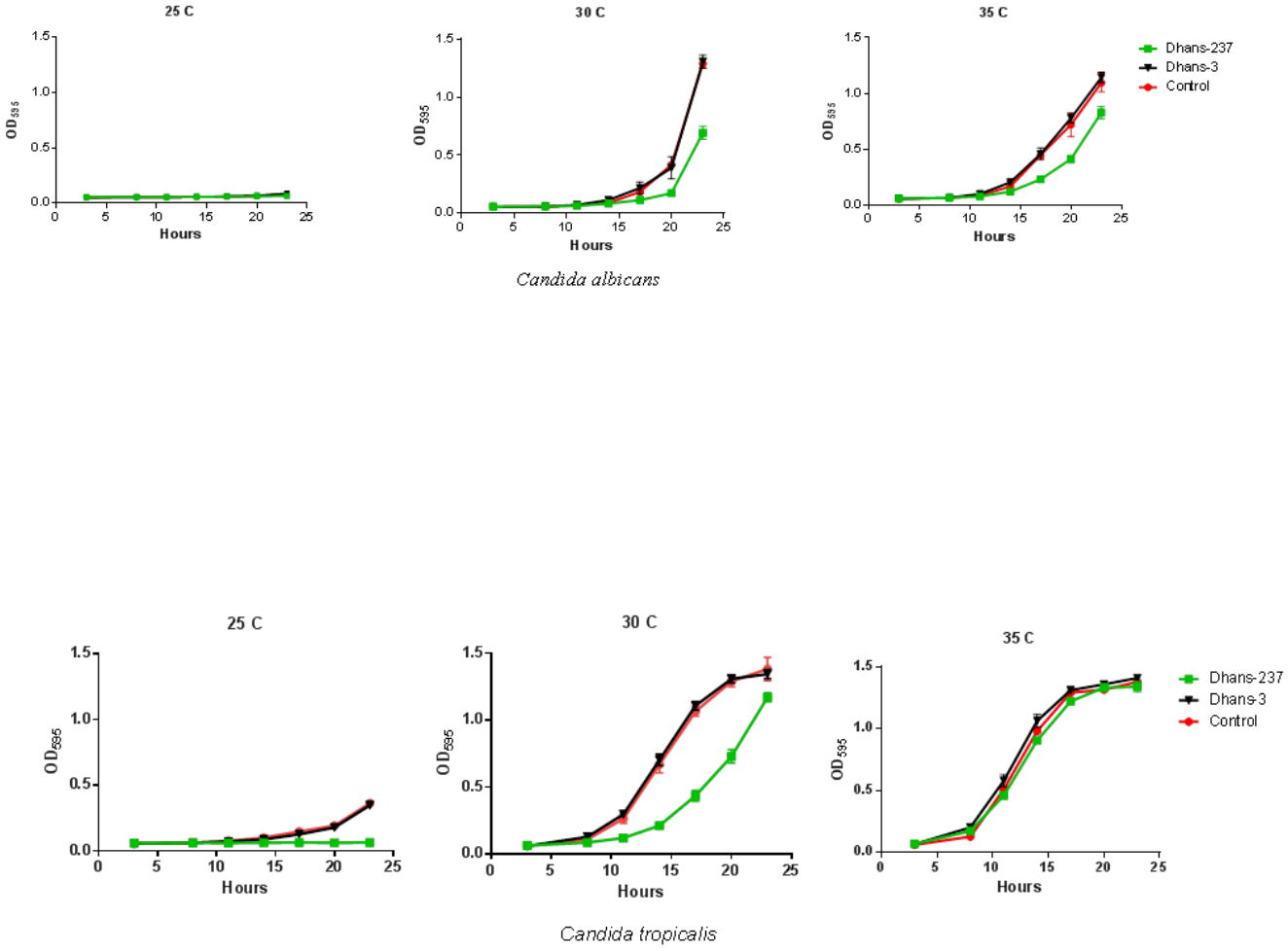

Figure 3-2 Influence of Dhans-237 killer toxin on the growth kinetics of *C. albicans* and *C. tropicalis* over 24 hours

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Conclusions

Our first study "Isolation of yeast and molds from different types of cheese collected from local markets, Lincoln, Nebraska, USA in 2012-2013" provides an overview of the fungi present in a variety of commercially-available cheeses. This study confirmed that cheese contains a diversity of molds and yeasts, and these vary with the type of cheese. More than 50% of cheese found in Lincoln, Nebraska, contained the yeasts *Debaryomyces hansenii* and *Galactomyces geotrichum*, and *P. roqueforti* was a frequently isolated mold in cheese. *D. hansenii* and *P. roqueforti* do not pose any safety issues. Some yeasts and molds were isolated only one time, but have been reported to cause infection and produce mycotoxins. In order to prevent the infection from these yeasts and molds, sources of contamination should be identified and addressed. Likewise, production of mycotoxins by molds in the cheese environment should be studied.

Our second study "The effect of *Debaryomyces hansenii* killer toxin against *Candida albicans* (SC5314) and *Candida tropicalis* (NRRL-10985)" demonstrated that approximately half of the *D. hansenii* strains isolated from different types of cheese have killer activity against *C. albicans* and *C. tropicalis*. Killer toxin activity differs among the *D. hansenii* strains. Killer activity of *D. hansenii* toxin against *C. albicans* and *C. tropicalis* is higher at low temperature and low pH but as temperature and pH increase killer activity decrease. Killer toxin activity was detected even at 35 C against *C. albicans* but not against *C. tropicalis*. *C. albicans* was more sensitive to killer toxin than *C. tropicalis* at high temperature while *C. tropicalis* was more sensitive than *C. albicans* at low temperature. The results confirmed that *D. hansenii* isolated from cheese

demonstrated killer activity against *C. albicans* and *C. tropicalis*, and the same killer toxin from *D. hansenii* can act differently in different species, temperature and pH condition. We found that killer toxin produced by Dhans-237 strain isolated from Italian Bel Paese cheese has activity at higher temperature, which may have medical application to inhibit the growth of *Candida* species.

Future directions

Killer yeasts have a large biodiversity, in terms of molecular characteristic, genetic determinants, spectra of action and mechanisms toxin actions. Most of the killer toxins have only one subunit but some killer toxins have two or three subunits (Liu et al., 2013). Some killer toxin genes are located in dsRNA virus in yeast cells, some in linear dsDNA plasmids and some are in chromosomal DNA (Magliani et al., 1997), but the clear genetic basis of killer systems of toxin produced by our *D. hansenii* strains and molecular characteristics are unknown. Similarly, killer toxins kill sensitive cells through various mechanisms, whether by causing ion leakage, inhibition of DNA synthesis, cell cycle arrest in G1, inhibition of DNA replication, or inhibition of glucan synthesis. *D. hansenii* killer toxin has been studied in few strains, and it is unknown which modes of action may exist, unlike the case in *S. cerevisiae, Williopsis* and other yeasts. Many results have shown that cell walls are a first target site for most killer toxin activity and different components of cell walls can be the receptors for killer toxin (Peng et al., 2010). Killer toxin produced by *S. cerevisiae, Kluyveromyces phaffii, W. saturnus* var *mrakii* strain MUCL41968, and *Brettanomyces* species kill their sensitive yeast cells by binding primary receptor 1,->6- β -D-glucan. After binding to the primary receptor in cell wall of

the sensitive cells, the killer toxin translocate to the secondary receptor in plasma membrane. Work on a single isolate reports 1->6- β -D-glucan as the binding site for *D. hansenii* killer toxins (Santos et al. 2002), but it is unknown whether *D. hansenii* produces a single toxin or several (as does *S. cerevisiae*). Similarly we do not know the inhibitory effect of proteoolytic enzymes over *D. hansenii* killer toxin. It has been well documented that *Saccharomyces cerevisiae* killer strains secrete killer toxins that are lethal to sensitive strains of same or related yeast species (Marquina et al., 2002). But we do not know whether different strains of *D. hansenii* have killer toxin activity against each other or not. However, there are still many open and unresolved questions regarding killer toxins of *D. hansenii.* So our future study will be focus on.

1. Genetic basis of killer systems of toxin produced by our *D. hansenii* strains
2. To determine the mode of action of *D. hansenii* killer toxin.
3. Assay to determine the effect of proteolytic enzyme over *D. hansenii* killer toxin.
4. Killer activity of *D. hansenii* strains against each other.

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